

New Technologies for Quantification of Protein Copy Number and Protein- Protein Interactions: From Single Plant Cells to Organelles

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Abstract

Absolute quantification of cell-to-cell variability arising from the stochastic nature of gene expression is concealed within bulk measurement approaches. While microfabricated technologies for single-cell biology have revealed and advanced our understanding of this heterogeneity, limitations remain in transforming this progress for plant biology. Therefore, this thesis provides single plant cell proteomic profiling technologies to challenge conventional bulk cell analysis to further our knowledge in biological variability.

Section one details the establishment, of the first known, miniaturised technology undertaking absolute protein quantification in single plant cells and organelles. Fabricated as an array of sub-nanoliter assay chambers containing micro-printed antibody spots, permitting multiplexed proteomic pull-down assays subsequent to the lysis of isolated protoplasts or organelles. Preliminary evaluation, employing a bi-molecular assay to assess fluorescent gene expression in protoplasts, presents novel experimental insight towards synergistic detection advantages through volume reduction coupled with single-molecule fluorescence microscopy. To demonstrate the quantification of unlabeled proteins a tri-molecular assay is developed for Rubisco, providing a new single-cell perspective towards Rubisco expression during mesophyll protoplast development.

Section two challenges the restrictions of antibody affinity and experimental throughput, by merging techniques to produce novel technologies. Initially, a coverslip unifying pull-down assay within a microwell array was developed to overcome throughput limitations and is evaluated by quantifying p53 expression in mammalian cell lines. While the pull-down assay faithfully reproduces the precision in quantitative measurements concerning epi-fluorescence microscopy, a more relevant tool, a secondary standard for quantitative fluorescence microscopy is created. Through ratiometrically calibrating chloroplast autofluorescence against the absolute fluorescent protein content of three transgenic cell lines.

These technologies permitted measurements, over six orders of magnitude for organelle, plant, and mammalian protein copy numbers, examined in Section three. Suggesting that unperturbed protein abundance does not scale with absolute expression noise, consequential to a linear relationship between the measured variance and mean.

Declarations Page

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Declaration of Originality

I certify that the work presented in this thesis is my own original work except where referenced otherwise.

For Izzy

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-Tom Mickleburgh

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List of Symbols and Abbreviations

2DE	Two-dimensional gel electrophoresis
4-HPPD	4- Hydroxyphenylpyruvate dioxygenase
ARC	Accumulation and replication of chloroplasts
AF	Autofluorescence
AF488	AlexaFluor 488
AF 647	AlexaFluor 647
APTES	(3-aminopropyl) triethoxysilane
ARR	Arabidopsis response regulator
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
cpDNA	Chloroplastic deoxyribonucleic acid
CE	Capillary electrophoresis
CFDA	Carboxyfluorescein diacetate acetoxymethyl ester
CFD	Computational fluid dynamics
CF	Carboxyfluorescein
CIB	Chloroplast isolation buffer
Da	Daltons
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FF _p	Fano Factor of protein distribution
FIJI	Phi Gamma Delta
FPP's	Fluorescent protein fusions
FP	Fluorescent proteins
FWHM	Full width half maximum
GFP	Green fluorescent protein
HLI	High light intensity

K	Shape parameter ascribed by a gamma distribution fit
kDa	Kilodaltons
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
m	steady-state mRNA content
M	Molar Concentration
MAC	Microfluidic antibody/ <i>affinity</i> capture
MDM2	Mouse double minute 2 homolog
MLI	Medium light intensity
mPEG	Methoxy polyethylene glycol
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NA	Numerical aperture
NPSN12	Novel plant SNARE 12
NSB	Non-specific background
ND: YAG	Neodymium-doped yttrium aluminum garnet
p	Steady-state protein content
p53	Tumor protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PLL	Poly L lysine
PVA	Poly (vinyl alcohol)
RCA	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase
Re	Reynolds number
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulfate
SCP	Single cell proteomics
SMC	Single molecule count

SMLM	Single molecule localization microscopy
SNR	Signal to noise ratio
SPR	Surface plasmon resonance
SSC	Saline-sodium citrate
TIRF	Total internal reflection fluorescence
UV	Ultraviolet
WAVE	FPP's for plant cell membrane compartment proteins
W5	Wash buffer
YFP	Yellow fluorescent protein
μ Fluidic	Microfluidic
μ TAS	Micro Total Analysis System
σ	Standard deviation
σ^2	Variance
μ	Mean
σ/μ	Coefficient of variation
σ^2/μ	Fano Factor
k_1	mRNA creation rate
k_2	Protein creation rate
γ_1	mRNA degradation rate
γ_2	Protein degradation rate
θ	Scale parameter ascribed by a gamma distribution fit

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List of Equations

Chapter 1.

Introduction: Micro Total Analysis Systems for Single Cell Proteomics

$$\phi = \frac{h\nu_{EM}}{h\nu_{EX}}$$

1.1. Quantum Yield (ϕ). A measurement of the fluorescence emission efficiency calculated by the ratio of photons emitted ($h\nu_{EM}$) to the number of photons absorbed ($h\nu_{EX}$) for a given fluorophore.

$$T = \frac{P}{P_0}$$

1.2 Transmittance (T). A ratio of the measured transmitted light power (P) to the intensity of the incident light (P_0)

$$A = -\log(T)$$

1.3 Absorbance (A). The logarithm of the reciprocal of the transmittance, a measurement of the capacity of a substance to absorb light.

$$\epsilon = \frac{A}{(d)(C)}$$

1.4 Beer-Lamberts Law. A measurement of the relationship between absorbance and the concentration of an absorbing species. This relationship is made possible through a solute's unique molar extinction coefficient (ϵ). This quantity is measured by dividing the absorbance (A) from the product of a known concentration of solute (C) and optical path length (d), usually defined by the width of the cuvette implemented.

$$A = \varepsilon(d)C = \log \frac{P_0}{P}$$

1.5 Substituting Transmittance in Beer-Lamberts Law. Assessing the relationship of the Beer-Lamberts Law, presented in equation 1.4, by replacing absorbance (A), seen in equation 1.3, with transmittance (T), described by equation 1.2.

$$P_F = \phi (P_0 - P)$$

1.6 Fluorescence Intensity. The measured fluorescence intensity of a distribution of fluorophores is representative of the power of the emitted fluorescence (P_F) from a sample. As this is equal to the amount of light absorbed by the fluorescent sample ($P_0 - P$) multiplied by the measured quantum yield (ϕ), previously presented in equation 1.1.

$$(A) \quad P_F = \phi P_0 (1 - e^{-\varepsilon d C})$$

$$(B) \quad P_F = \phi P_0 (\varepsilon d C)$$

1.7 A & 1.7 B Quantitative Fluorescence. By substituting the explanation of absorbance from the Beer-Lambert law, in equation 1.5, for the light absorbance of the fluorescent sample, equation 1.6. Followed, for practical purposes, a reduction of the equation by deeming the first term significant at small values of ($\varepsilon d C$), presenting the proportionality of emitted fluorescence (P_F) to the number of fluorophores present within a solution.

$$FF_p = \frac{\sigma^2}{\mu}$$

1.8 The Fano Factor (FF_p) of an arbitrary stochastic system reveals the deviations from a Poissonian behavior where $FF_p = 1$. It is quantified as the variance (σ^2) normalized by the mean (μ).

$$\eta^2 = \frac{\sigma^2}{\mu^2}$$

1.9 Quantification of Noise. Noise within this thesis has been quantified as the squared coefficient of variation, that is the variance (σ^2) normalized by the square mean (μ^2).

$$\eta^2 = \frac{1}{\mu}$$

1.10 Gaussian Probability Distribution. As the number of molecules per each measurement in an experimental trial proceeds to infinity, the variation around the mean of these measurements approaches 0. This behavior is rooted in ergodic dynamics combined with the central limit theorem.

$$\eta^2 = \eta_i^2 + \eta_e^2$$

1.11 Composition of Noise. The total noise measurement (η^2) of protein expression is a summation of both the intrinsic noise (η_i) contributions and extrinsic noise (η_e) contributions.

$$P(x = k) = e^{-\mu} \frac{\mu^k}{k!}$$

1.12 Poissonian Distribution Probability Density Function. A prediction of the number of events (translational bursting/ single cell trapping) occurring ≥ 0 times in an interval. The probability of observing (k) events with an average number of events (μ) expected in time.

$$P(x) = \frac{x^{\alpha-1} e^{-x/\beta}}{\Gamma(\alpha) \beta^\alpha}$$

1.13 Gamma Distribution Probability Density Function. Whereby x is average protein copy number and the distribution is uniquely determined by the shape parameters K and scale parameter θ .

Chapter 2. Basic Principles & Techniques

$$Re = \frac{L V_{avg} \rho}{\mu_v}$$

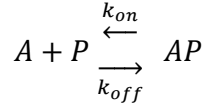
2.1 Reynolds Number (Re). A unit-less quantity, which relates the fluid density (ρ), average velocity (V_{avg}), and viscosity (μ_v) within the hydraulic diameter (D) for quantification of the turbulence in fluid flow.

$$R_{Fluidic} = \frac{\Delta P}{Q}$$

2.2 Fluidic Resistance. The resistance to flow ($R_{Fluidic}$) is determined by the fluid and its conduit, calculated as a ratio of pressure drop (ΔP) and the volumetric flow rate (Q).

$$\frac{\partial \phi}{\partial t} = D \frac{\partial^2 \phi}{\partial x^2}$$

2.3 Fick's Law of Diffusion. The effective diffusion coefficient (D), can model the molecular rate of the diffusion process, in a dimensional concentration (ϕ), when the location (x) and time (t) have been specified.



2.4 Bimolecular Interactions. The interactions of a protein (P) and an antibody (A) undergoes dynamic binding events of collisions (k_{on}) and dissociation (k_{off}). Upon reaching equilibrium portions of A and P will create a complex (AP), while portions will be free.

$$\frac{d[AP]}{dt} = [A] \cdot [P] \cdot k_{on} - [AP] \cdot k_{off} = 0$$

2.5 Mass Action Law. Is representative of the interactions of a protein (P) and an antibody (A) at equilibrium, that is the concentrations do not change, setting the interactions equal to zero.

$$[A] \cdot [P] \cdot k_{on} = [AP] \cdot k_{off}$$

2.6 Thermodynamic Equilibrium. As equation 2.5 is set to zero, the equation can be rearranged defining the dynamic equilibrium of the complex (AP) and free portions of A and P .

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[A] \cdot [P]}{[AP]}$$

2.7 Equilibrium Dissociation Constant (K_D). Rearranging equation 2.6, when the bimolecular interaction between A and P is at equilibrium, produces a ratio of K_{on} to K_{off} , which is the definition of K_D and relates to the concentration of antibody with respect to the antibody's affinity (i.e. higher affinity of an antibody to a protein is descriptive of a lower K_D value).

$$[AP] = \frac{[A] \cdot [P]}{K_D}$$

2.8 Antibody Affinity. Equation 2.7 can be rearranged to present evidence that the smaller the K_D value, the higher the affinity of complex formation $[AP]$ of the antibody $[A]$ for its antigen or protein $[P]$.

$$F_{bound} = \frac{[AP]}{[A + P]}$$

2.9 Fraction of Protein Bound. The ratio of complex formation is a measurement of the fraction of protein bound (F_{bound}). This can be a direct readout of either the amount of protein (P) in the system when the number of (A) is constant or the number of antibodies (A) in the system when the number of (P) is constant.

$$\theta_c = \sin^{-1} \left(\frac{n_2}{n_1} \right)$$

2.10 Snell's Law. As light passes through one medium's refractive index (n_1) into another medium's refractive index (n_2) incident light will be reflected back into the original medium (n_1) if the refractive index relationship satisfies a critical angle (θ_c) equal to or greater than 90° .

$$I(z) = I_0 e^{-z/d}$$

2.11 Penetration Depth of the Evanescent Wave. The penetration depth of incident light into another medium (n_2) satisfying total internal reflection conditions, presented in Equation 2.10, demonstrates exponential decay. The depth of this incident light can be calculated by (d) representing penetration depth, $I(z)$ is the light intensity at a perpendicular distance (z) from the interface (n_1, n_2) for a given light intensity (I_0).

$$\bar{\mu} = \frac{1}{n} \sum_{i=1}^n x_i = \frac{x_1 + x_2 + \dots + x_n}{n}$$

2.12 Sample Mean. The mean ($\bar{\mu}$) is calculated by the summation of all values (x_i) in the sample divided by the number of values in the sample (n) and is an estimate for the population mean (μ).

$$SS = \sum (x_i - \bar{\mu})^2$$

2.13 Sum of Squares (SS). A measurement of the dispersion of observations around a sample mean, calculated by subtracting the mean ($\bar{\mu}$) from an observation value (x_i) and squaring this deviate, followed by summing these squared deviates.

$$v^2 = \frac{\sum (x_i - \bar{\mu})^2}{(n - 1)}$$

2.14 Sample Variance (v^2). A measurement of the spread of values with respect to the mean, calculated through dividing the sum of squares, presented in equation 2.13, by the number of values in the sample (n-1).

$$\sigma = \sqrt{\frac{\sum (x_i - \bar{\mu})^2}{(n - 1)}}$$

2.15 Sample Standard Deviation (σ). A measurement of dispersion in its original units, not squared units, performed by taking the square root of equation 2.14.

$$\sigma_x^- = \frac{\sigma}{\sqrt{n}}$$

2.16 Sample Standard Error (σ_x^-). An assessment of how far the sample mean ($\bar{\mu}$) is likely to be from the population mean (μ), achieved by taking the standard deviation and dividing the squared number of values in the sample ($\sqrt{n}$).

$$\eta = \frac{\sqrt{\frac{\sum (x_i - \bar{\mu})^2}{(n - 1)}}}{\bar{\mu}}$$

2.17 Coefficient of Variation (η). A summary of the variation as a percentage of the total, independent of units, calculated by dividing equation 2.14, the sample standard deviation, by equation 2.12, the sample mean.

$$\frac{1}{n} \sum_{i=1}^n (x_i - \bar{\mu})^r$$

2.18 Probability Distribution Shape. Probabilities of finding values (x_i) which would exist within a population based on sample measures of location ($\bar{\mu}$) and variation (v^2) are descriptive of moments about the mean. Where the exponent r is described as the r^{th} moment. The first moment is equal to the mean. The second moment about the mean is variance. The third moment is related to the skewness and the fourth moment is related to the kurtosis of the distribution.

$$P(X = x) = p[a \leq x \leq b] = \int_a^b f(x)dx = 1$$

2.19 Probability Density Function. The likelihood of finding a value (x) in a continuous distribution that represents the measured variable (X) between two points (a and b) is predicted through a probability density function defined as $P(X)$. The integral of the probability density function is 1, which arises from the property of discrete distributions in which the sum of all the probabilities must equal one.

$$(A) \quad A^2 = -N - S$$

$$(B) \quad F_i = \frac{x_i - \bar{\mu}}{\sigma}$$

$$(C) \quad S = \sum_{i=1}^N \frac{(2i-1)}{N} [\ln(F(F_i)) + \ln(1 - F(F_{N+1-i}))]$$

2.20 A B and C Anderson Darling Test. Assessment for the quality of a distributional fitting to sample data. Defined, in equation 2.20 A, by assessing the test statistic (A) for a given distribution with a sample size (N) against that of the given critical values assigned for a univariate distribution (e.g. normal distribution, log-normal distribution, Weibull distribution and exponential distribution). S is solved, in equation 2.20 C, by the cumulative distribution function (F) of the specified distribution and the standardised values in the univariate data distribution (F_i), presented in equation 2.20 B.

$$cov(X, Y) = \frac{1}{N} \sum_{i=1}^N (x_i - \bar{\mu}_x) (y_i - \bar{\mu}_y)$$

2.21 Covariance in Bivariate Sample Data. Relationships in bivariate data can be described by their association, that is if X in the i^{th} individual changes does the Y variable for the same individual tend to change. This association is measured by the covariance, where sample mean ($\bar{\mu}$) is denoted by the x or y subscript, and i denotes an individual in the data set.

$$r = \frac{1}{N-1} \sum_{i=1}^N \left(\frac{(x_i - \bar{\mu}_x)}{\sigma_x} \right) \left(\frac{(y_i - \bar{\mu}_y)}{\sigma_y} \right)$$

2.22 Pearson Correlation Coefficient (r). A value of the bivariate data association presented in equation 2.21, normalized to scores between -1 and 1 to allow comparison between the amount of association among samples with different values. Where sample mean ($\bar{\mu}$) and standard deviation is denoted by the x or y subscript, and i denotes an individual in the data set.

$$y = a + b_{yx}x$$

2.23 Linear Regression. The process of linear regression is generating a linear line of best fit for a given distribution of bivariate data to represent the correlated relationship between X and Y. In which X and Y represent the variables, (a) is the y-intercept, and (b_{yx}) is the regression coefficient. The regression coefficient represents the rate of change in y as a function of a change in x.

$$(A) \quad R^2 = 1 - \frac{SS_{res}}{SS_{tot}}$$

$$(B) \quad R_{adj}^2 = 1 - \frac{s^2_{res}}{s^2_{tot}}$$

2.24 Coefficient of Determination (R^2). Describes the percentage of the response variable variation that is explained between the bivariate data through examining the points on the graph and their distance to the regression line, a best fit line defined by equation 2.23. This distance examination is performed by dividing the residual sum of squares (SS_{res}) calculated by subtracting the model estimates from the actual data by the total sum of squares (SS_{tot}), the latter being proportional to the total variance of the data, both sum of square values are calculated using equation 2.13. Comparison of the suitability for regression line fits can be performed by adjusting the coefficient of determination through equation 2.24 B.

$$x_i(X) = \frac{x_i(X)}{\mu(X)}$$

2.25 Data Normalisation. The ability to decouple the units of measurement to compare data sets, is performed by each measurement (x_i) in a data set (X) being divided by the mean value (μ) of the data set. A concept previously presented in equation 2.17.

$$(A) \quad x_i(X) = x_i(X) - \mu(X)$$

$$(B) \quad z_i = \frac{x_i(X) - \mu(X)}{\sigma(X)}$$

2.26 Standardised z-score. The ability to acquire a z-score (z_i) is achieved by taking equation 2.26 A and dividing this by the standard deviation of the sample. This transformation produces comparable distributions through scaling by the standard deviation, allowing independence from units. The mean value is 0 while the standard deviation is 1 for all standardised samples.

$$F = \frac{\eta(X)}{\eta(Y)}$$

2.28 F-test. An assessment of the precision based upon the quotient of the coefficient of variation (COV) between two measurement platforms. A value of 1 presents equal dispersion around the average measurements, therefore high precision.

Chapter 3. Single Cell to Organelle Rubisco Quantification

$$\frac{\text{mass concentration (g L}^{-1}\text{)}}{\text{molar mass (g mol}^{-1}\text{)}} \times [\text{Avogadro's Constant (6.023E}^{23}\text{mol}^{-1}\text{)}] = \frac{\text{molecules}}{L}$$

3.1 Calculating the Number of Molecules. By calculating the number of moles L⁻¹ by taking the ratio of the mass concentration (g L⁻¹) with the known molar mass (g mol⁻¹ or Da). Through multiplying the number of moles L⁻¹ with the Avogadro constant (6.023 x10²³ mol⁻¹) the number of molecules per L is calculated.

$$\frac{\text{molecules}}{L} \times \text{Chloroplast Volume (3.3E}^{-14}\text{)} = \text{protein molecules per chloroplast}$$

3.2 Number of Molecules per Chloroplast. Through multiplying the number of molecules per L, calculated from *Equation 3.1*, with the average chloroplastic volume. A back of the envelope calculation of molecules per chloroplast can be calculated.

$$AIC = 2 * \ln L + 2 * k$$

3.3 Akaike Information Criterion (AIC). To estimate the quality of a model relative to other models, assessment is achieved by taking the maximum value of the likelihood function for a model (L) coupled with the number of estimated parameters in the model (k), presenting a means for model selection.

Chapter 5.

A Reduced Volume Microwell Array

$$B_{Ag} = \frac{Ab}{Ab + K_d} \times 100$$

5.1 Estimate of Percent Antigen Bound (B_{Ag}). Estimates the percentage of antigen bound upon manipulating the concentration of a capture antibody (Ab) with a known dissociation equilibrium constant (K_d).

$$B_{Ag} = \frac{(Ag)(Ab)}{K_d (6.023 \times 10^{23})(V)(Ag)} \times 100$$

5.2 Estimate the Antigen Bound with Assay Volume Manipulation. The percentage of antigen binding events in a predetermined assay volume (V) can be estimated.

$$B_{Ag} = 0.5 = \frac{Ag}{Ag + K_d}$$

5.3 Estimating K_d from Saturation Binding Curves. The dissociation equilibrium constant is estimated by alterations in antigen concentration presenting a fractional saturation (B_{Ag}) of 0.5

$$\% \text{ Disagreement} = \frac{\text{Measured} - \text{Predicted}}{\text{Measured}} \times 100$$

5.4 Evaluating Percent Agreement. The percent disagreement evaluation between the mathematically estimate values (B) and the experimentally measured values (A).

Chapter 6.

Quantitative Fluorescence Microscopy

$$(x, y) = I(x, y) \cdot D(x, y) \cdot F(x, y) \cdot T(s)$$

6.1 Pixelated Image Product Distribution. The product distribution of a pixelated image (P) area (x, y) acquired by a fluorescent microscope is composed of the illumination distribution (I) the detection efficiency (D) and the Poisson noise generated from the fluorescent molecule distribution (F). Coupled with the time of image exposure [T(s)]

$$F_{obj} = \sum_{i=1}^{i=N_{obj}} F_{obj_i} - N_{obj} \frac{\sum_{j=1}^{j=N_{bkg}} F_{bkg_j}}{N_{bkg}}$$

6.2 Epi-Fluorescence Background Subtraction. To acquire the true fluorescence signal of an object (F_{obj}) background subtraction must be performed. Whereby the summated fluorescence intensity (F) of all pixels within the object (i) or background pixels (j) within the background image (bkg) have an equivalent number of pixels (N) under constant (I)(T(s)) and (D) see Equation 6.1.

$$\frac{\mu(X)_{MAC}}{\mu(Y)_{MAC}} = MAC_{RM}(X, Y) \quad \text{and} \quad \frac{\mu(X)_{EPI}}{\mu(Y)_{EPI}} = EPI_{RM}(X, Y)$$

6.3 & 6.4 The Ratio of Means. A ratiometric comparison of average measurements (μ) by microfluidic antibody capture chip (MAC) or epi-fluorescence microscopy (EPI) for two differing cell lines (X and Y). Presenting a ratio of the means measurement for MAC chip measurements [$MAC_{RM}(X, Y)$] and fluorescence microscopy measurements [$EPI_{RM}(X, Y)$].

$$\frac{EPI_{RM}(X, Y)}{MAC_{RM}(X, Y)} = \% \text{ Method Equivalence}$$

6.5 Assessment of Method Concordance. Through taking the quotient of the ratiometric means for two measurement platforms, such as epi-fluorescence microscopy (EPI_{RM}) and MAC single molecule quantification (MAC_{RM}). This present a measurement of accuracy (% Method Equivalence)

$$P(x) = [1.21 \times 10^4] \left[\frac{F_x}{F_{chl}} \right] + [9.6 \times 10^3]$$

6.6 Absolute Quantification in Epi-Fluorescence. After background subtraction upon autofluorescent chloroplasts (F_{chl}) and the cell to be quantified (F_x) a ratiometric comparison is measured ($\frac{F_x}{F_{chl}}$). Upon placement of ($\frac{F_x}{F_{chl}}$) into a calibrated linear fit ($y=mx+ b$) calculated from absolute copy number fluorescent intensity standards. The absolute copy number (x) can be solved for the unknown fluorescent intensity of a cell (F_x).

Chapter 7.

Sources of Noise in Gene Expression

$$x ; Gamma (K, \theta); p(x) = \frac{x^{K-1} e^{-x/\theta}}{\Gamma(K)\theta^K}$$

7.1 Gamma Distribution Probability Density Function. Whereby x is average protein copy number and the distribution is uniquely determined by the shape parameters K and scale parameter θ .

$$K = \frac{\gamma_2}{k_1} \quad \& \quad \theta = \frac{k_2}{\gamma_1}$$

7.2 Biochemical Rates of Gamma Distribution Parameters. The shape parameter (K) is descriptive of protein burst timing, the ratio of protein degradation (γ_2) to mRNA creation(k_1). While the scale parameter (θ) is descriptive of the protein burst size, the ratio of protein creation (k_2) to mRNA degradation (γ_1)

$$K\theta = \mu \quad \& \quad K\theta^2 = \sigma^2$$

7.3 Mean and Variance of the Gamma Distribution. The shape (K) and scale (θ) parameters of the gamma distribution are descriptive of both the mean (μ) and the variance (σ^2) measured within a distribution.

$$\eta^2 = \frac{\sigma^2}{\mu^2} \approx \frac{K\theta^2}{(K\theta)^2} = \frac{1}{K} = \frac{k_1}{\gamma_2}$$

7.4 Relation of Burst Timing with Expression Noise. The inverse of the shape parameter (K) can be approximated from the total noise in a protein distribution (η^2)

$$\text{FF}_p = \frac{\sigma^2}{\mu} \approx \frac{K\theta^2}{K\theta} = \theta = \frac{k_2}{\gamma_1}$$

7.5 Relation of Burst Size with Noise Strength. The noise strength or Fano Factor (FF_p) can be approximated by the scale parameter (θ) of a distribution. This scale parameter is descriptive of protein burst size, see equation 7.2.

$$\eta^2 \approx \frac{1}{\mu}$$

7.6 Intrinsic Noise Limit. The intrinsic noise in protein expression (η_i) can be approximated from the inverse of the mean (μ).

$$\mu = K\theta; \sigma^2 = K\theta^2; \rightarrow \sigma = \left(\frac{1}{\sqrt{K}}\right)\mu \quad \therefore \quad \eta = \frac{\sigma^2}{\mu^2} = \frac{1}{K}$$

7.7 Scaling of Variance and Mean in Protein Expression. One possible explanation for the correlation between the variance (σ^2) and the mean (μ) in protein expression is the shape parameter (K) being constant.

$$m = \frac{k_1}{\gamma_1} \quad \text{and} \quad 1 \approx \frac{\sigma_m^2}{\mu_m}$$

7.8 Approximating mRNA in Single Gene Expression. mRNA steady-state levels (m) can be estimated as the creation of mRNA (k_1) normalized by the mRNA degradation rate (γ_1). Thattai et al approximated this steady state distribution as a Poissonian distribution.

$$p = \frac{k_1 \left[\frac{k_2}{\gamma_1} \right]}{\gamma_2} \quad \text{and} \quad \frac{\sigma_p^2}{\mu_p} = \frac{\left[\frac{k_2}{\gamma_1} \right]}{1 + \left[\frac{\gamma_2}{\gamma_1} \right]}$$

7.9 Approximating protein in Single Gene Expression. Protein steady-state levels (p) can be measured by the multiplication of the shape and scale parameter, see equation 7.7. This can be expressed as the scale parameter (θ) multiplied by the rate of mRNA creation (k_1) divided by the rate of protein degradation (γ_2). Thattai et al approximated this steady state distribution by the rate of protein creation (k_2) divided by mRNA degradation (γ_1). Divided by the protein lifetime (γ_2/γ_1).

$$CV^2_T = CV^2_E + CV^2_P + CV^2_R$$

7.10 The Additive Process for Total Noise in Protein Expression. The total noise measured in protein expression (CV^2_T) is a cumulative source of intrinsic noise, described as protein expression noise (CV^2_P) coupled with extrinsic noise arising from partitioning events (CV^2_R) and cell cycle noise (CV^2_E)

"I often say that when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts advanced to the state of Science."

-William Thomson (Lord Kelvin)
1883

Chapter 1

Introduction: Micro Total Analysis Systems for Single Cell Proteomics

This introductory chapter highlights the elements in microfluidics, fluorescence, and proteomics which have been interwoven to create a micro total analysis system, titled the Microfluidic Antibody Capture Chip. Techniques similar to this, allowing single-cell proteomic measurements are reviewed. Followed by a discussion on the practical implications of this approach towards our quantitative understanding of cell-to-cell variability. While these single-cell approaches are employed in a growing number of animal studies, a gap remains in their application for quantitative plant biology.

1.1 Introduction

Abiotic stress-resistant crops, cancer stem cells, and drug-resistant bacteria are examples of biological organisms that thrive in a variety of environmental and physiological conditions. Understanding this *modus operandi* of survival is achieved by implementing the language and principles of chemistry, facilitating measurements towards the molecules and chemical reactions that constitute cellular function. As the current knowledge of biology has progressed, the validity of biological models conceived by population-average measurements (e.g., Western Blot, 2D Page/MS, LC-MS/MS, ELISA) to explain cellular behaviour may present misleading or inaccurate interpretations [1,2]. A simple example of this misinterpretation, as shown in *Figure 1.1*, can be when an average measurement of 50% protein expression from a cell population arises from only half the cells presenting 100% expression or a 50% expression within the total cell population [2].

This deviation from accuracy derives from the ignorance of cell-to-cell variability, in part due to technical limitations. However, while these limitations gradually disappear due to advancements in single-cell biology, the novel and once overlooked molecular differences in single cells can be quantified, permitting greater accuracy within biological models

influencing our precision towards understanding absolute rates of biological processes, pharmacological responses, and the general architecture of cellular structures.

While an aspiring statistician, such as Harry Nilsson, wrote in a song that “one is the loneliest number that you will ever know” the technology applied for single cell measurements is far from lonely. Arising from a need to integrate multiple functional elements to produce a cell-in/answer-out system, highlighted by our previous review paper [3]. The first step in fabricating this system is the isolation of a single cell. Followed by the extraction of molecules or chemical products that constitute cellular function and finally a detection system to provide the answer. This introductory chapter highlights these critical elements (microfluidics, proteomics, and fluorescence detection) which have been interwoven to create a cell-in/answer-out Microfluidic Antibody Capture (MAC) Chip, a novel technology platform allowing insight towards plant proteomic measurements.

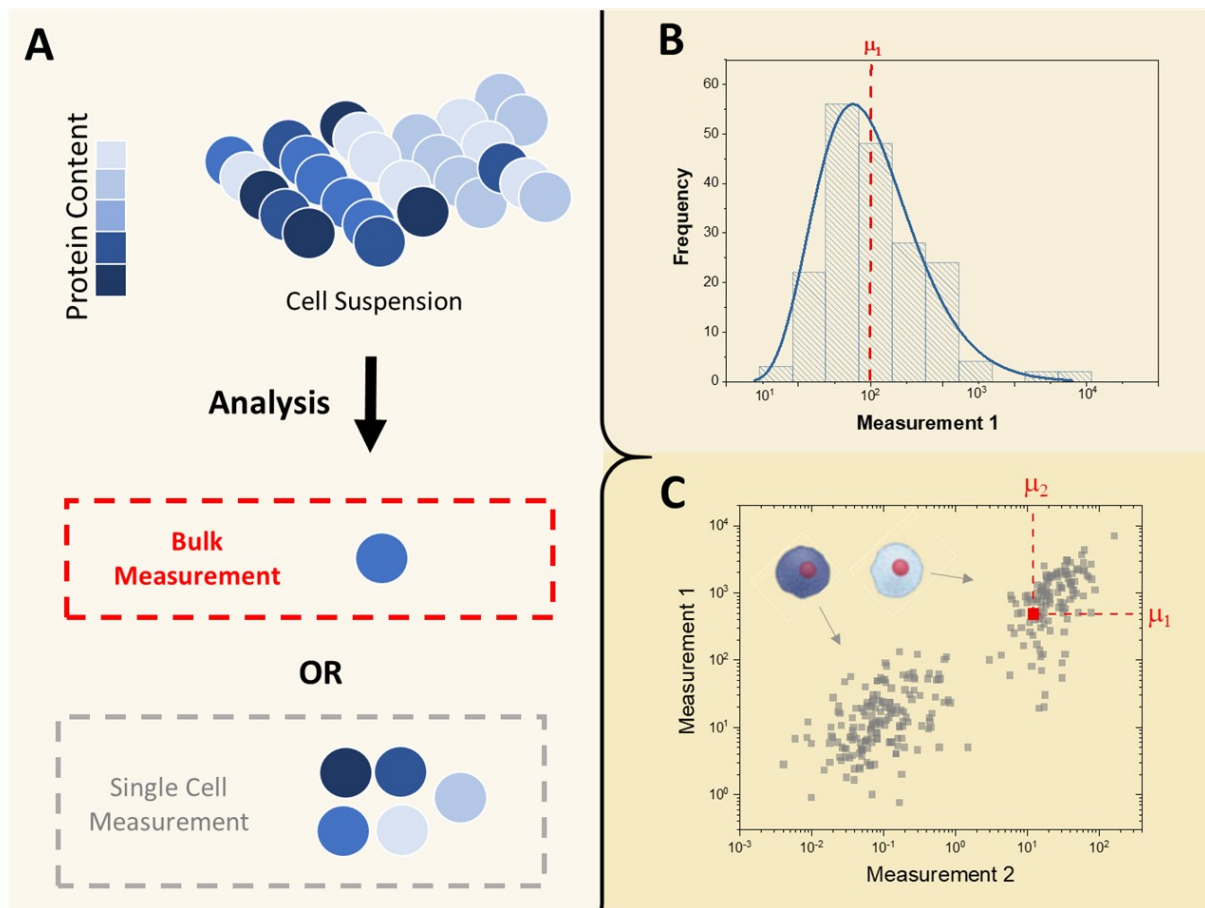


Figure 1.1 Ensembled Averages and Heterogeneity. (A) Measurements on a population of cells can be performed by bulk measurement or single cell measurement techniques. (B) The ensembled-average measurement (dashed line at μ_1) can mask information of population heterogeneity, which is revealed through the single-cell histogram data. (C) Through performing multiplexed measurements (cell size, division time, protein or cell surface marker expression) at single cell resolution different patterns of cellular heterogeneity may be identified. These patterns may be identified by visual or computational methodology.

1.2 Microfluidics

The science of precisely manipulating small amounts of fluid is the fundamental concept of microfluidics. Through the symbiotic relationship between this miniaturisation science and other scientific fields microfluidics has developed into an advantageous technology, in part due to the reduction of sample volumes and assay times. Evident by current advancements to take this efficient technology from university laboratories into commercial technologies through companies such as Fluidigm, Fluigent, RAB-Microfluidics, and Eksigent to name a few.

1.2.1 Advancements in Micro Total Analysis Systems

In the beginning, this microscale process existed in capillary format to achieve separation and detections with higher resolution and sensitivity in analytical devices (e.g., capillary electrophoresis, gas-phase chromatography, high-pressure liquid chromatography), demonstrated in 1975 by Terry et al. as the first miniature gas chromatographic analysis system was revealed [4]. After seeing the revolutions in chemical analysis, microfluidics was adapted into the field of molecular biology due to the genomic explosion of 1980, leading to the demand of analytical methods with higher sensitivity, resolution, and throughput. Around the 1990's the cold war ended, yet the threat of chemical and biological weapons still existed. Governmental funding bodies provided the academic stimulus to develop novel small-scale systems that could detect these threats [5]. Upon those governmental funds, the first micro total analysis system (μ TAS) was born, whereby a variety of functional elements for sample manipulation and handling are integrated into one microfluidic device [6]. During this past 30 years μ TAS has been integrated into biological and biomedical analysis creating niche areas of study. Single-cell biology is one of these resulting studies [7].

Microfluidic devices have become integrated within many other aspects of research, this is in part due to the variety of fabrication methods, as displayed in *Figure 1.2*, and a sufficient range of technological components that can be integrated. Allowing the creation of inexpensive fluidic, droplet, paper and electrokinetic devices driving the field to become exponentially vast since the μ TAS dawn in the 1990's [3]. To highlight a few of these applications, microfluidic devices presented at μ TAS 2017 show promise in the field of organic and medicinal chemistry synthesis, cancer and disease diagnostics, nanowire or nanoparticle synthesis, cancer and bacteria analysis, protein, and DNA analysis, also the fabrication of organs and blood-brain barriers on a chip. Through our groups novel

interrogation on plant cells, we were excited to add plant analytics to this list of applications [8].

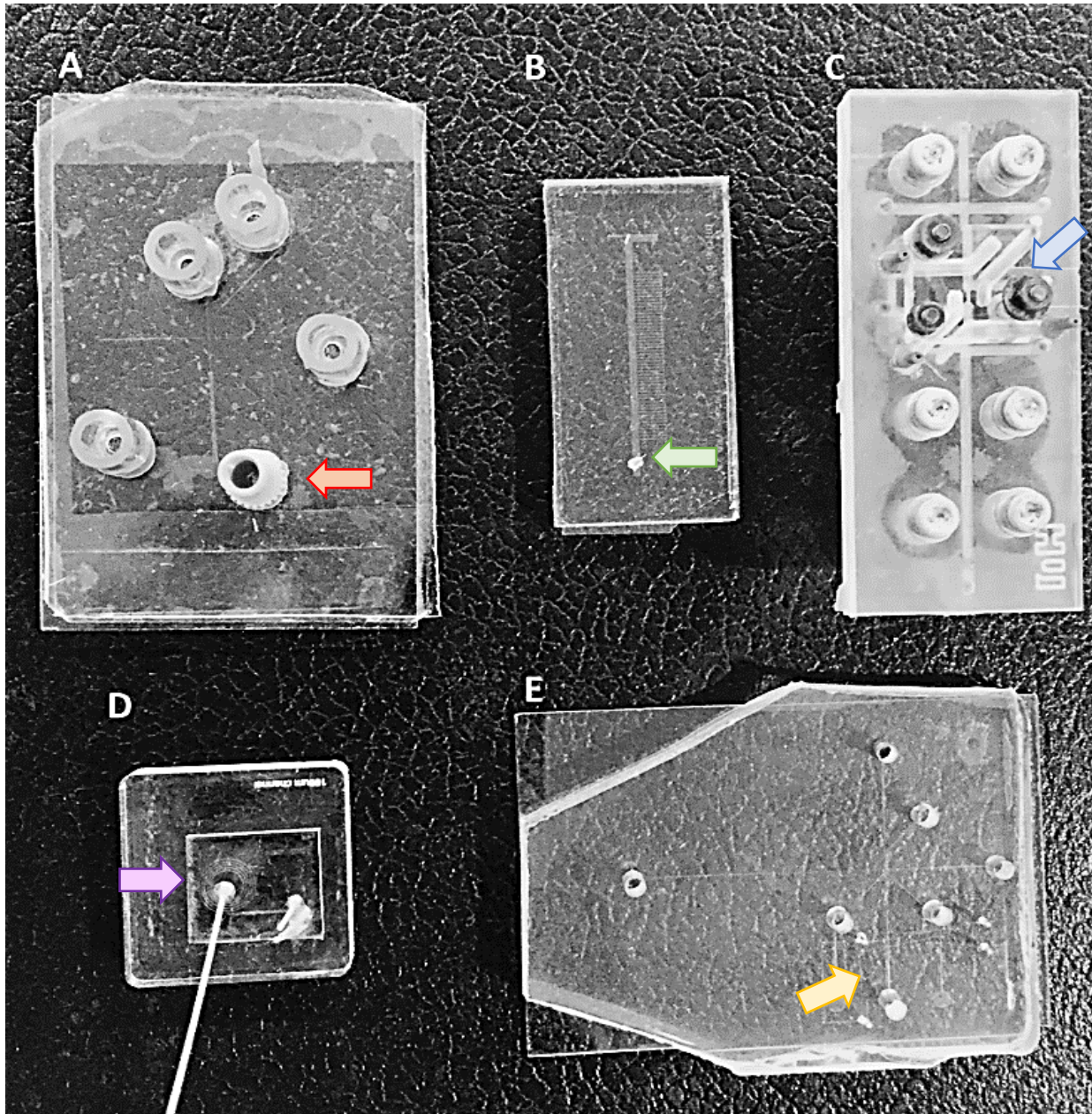


Figure 1.2 Fabrication Methods for μ Fluidic Devices. Over the last decade microfluidic device creation has rapidly changed through the advancements of microelectronics. (A) Originally, devices were fabricated through wet etching (the controlled addition of Hydrofluoric Acid) creating optically transparent, high aspect ratio structures. Large glass reservoirs ($100\mu\text{L}$) may also be attached with connections (red arrow) for syringe pumps allowing organic chemical manipulation. (B) PDMS, in combination with soft-lithography, became an important fabrication tool for microfluidic devices for single cell analysis and easily binds to glass, furthermore reservoir creation ($30\mu\text{L}$) could be achieved through biopsy punches (green arrow) allowing a reduction in fluidic volume. (C) PMMA a shatter resistant alternative to glass can be fabricated into a microfluidic device by laser ablation or hot embossing techniques, through integration of screws (blue arrow) or a thin layer of PDMS one can create valves allowing precise biological fluid extraction after sample concentration through ITP. (D) A combination of both PDMS for complex channel construction (spiral channels for particle separation/purple arrow) bound with PMMA for structural integrity. (E) Valves (yellow arrow) may also be created within a PDMS device through multilayer device construction, allowing fluidic control within the device, more details of this process are described in Figure 1.3.

1.2.2 Microfluidic Manipulation by Fabrication

Microfluidic systems offer three key advantages in single cell analysis through the isolation, extraction, and detection of biomolecules from single cells. In part due to both the fabrication of optimal single-cell dimensional channels and the implementation of polydimethylsiloxane (PDMS). PDMS is a low toxic, optically transparent material which is highly permeable to dioxygen and carbon dioxide. An ideal material for which to grow and observe single-cell characteristics. PDMS is an ideal material for the fabrication of a multitude of high-throughput hydrodynamic trapping structures, as proven by the Di Carlo group [10, 11, 12]. Both Quake and Mathies groups have exploited PDMS, due to its elastomeric properties, allowing valve creation [13, 14]. Valves a crucial foundation within the field of microfluidics are designed by producing a control valve layer on-top of a fluidic channel layer as shown in *Figure 1.3 A*. These valves allow isolation volumes for single cells ranging from tens of picoliters to tens of nanoliters [14]. Valves can also be implemented for three-state peristaltic pumping, as presented in *Figure 1.3 C*. A significant advancement not only for the preliminary step of single cell isolation but the subsequent chemical cytometry process within single-cell lysate [15].

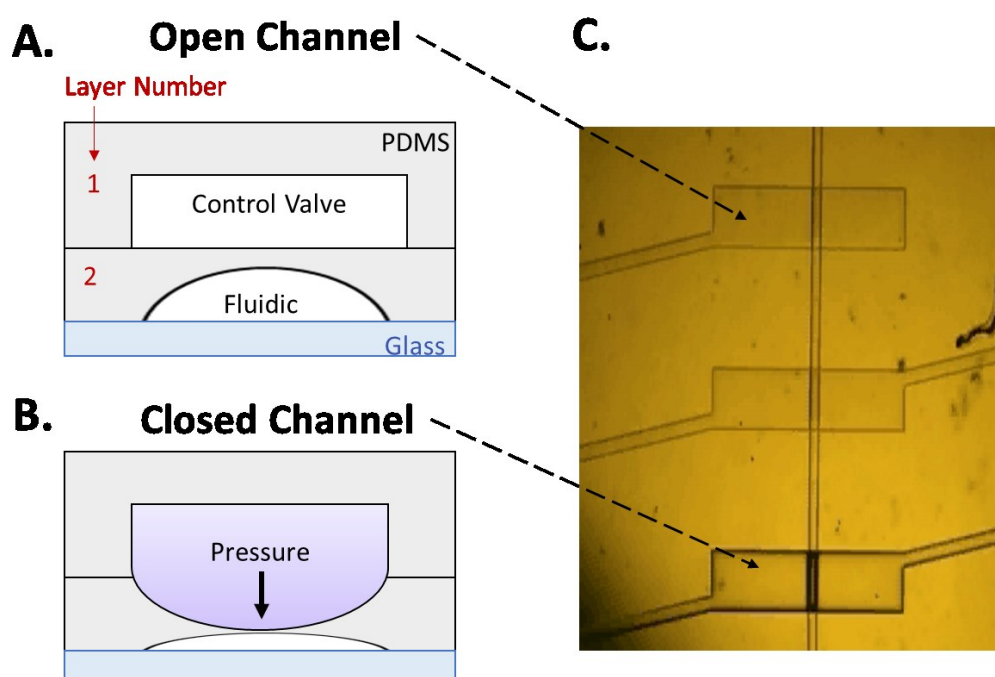


Figure 1.3 Integration of Microfluidic Valving. (A) Multilayer microfluidic devices composed of a control valve layer (1) and a fluidic layer (2) allow precise fluid manipulation or single cell trapping capabilities. (B) When pressure is exerted within the control valve, the fluidic layer collapses, actuating the closure of the fluidic channel. (C) A Brightfield image of the combined components with both open and closed channels. When these valves are actuated in a peristaltic wave motion, a precise fluidic control has been shown to be exerted within the fluidic channels.

Single Cell Isolation

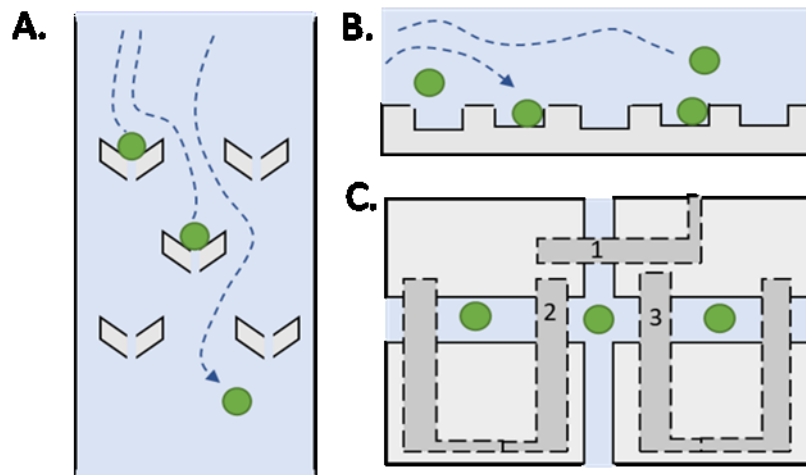


Figure 1.4 Single Cell Isolation Methods. A variety of isolation methods exist, some of these methods entail (A) hydrodynamic trapping, where cells encounter u-shaped traps that are biased to trap single cells. (B) Microwells are a method for isolating single cells from a suspension of cells. (C) The implementation of microfluidic valves (1,2,3), such as those previously presented in Figure 1.3, can also be applied to trap and isolate single cells.

1.2.3 Single Cell Isolation

Single cell isolation can be achieved through PDMS fabrication methods, as represented in *Figure 1.4*, or through the integration of alternative non-PDMS based technologies within the microfluidic channels [16]. Such as the implementation of magnetic or optical traps, allowing the precise placement and manipulation of single cells. Applications of electric fields within channels allow the trapping of single cells by dielectrophoresis (DEP) or acoustic methods. DEP influences cell patterning in microfluidic devices through the introduction of microelectrodes which produces a non-uniform electric field in which single cells move towards based on the polarity of the single cell [17]. Acoustic trapping is the creation of a standing surface acoustic wave allowing a non-contact way of introducing cell patterning within a microfluidic device. This standing surface acoustic wave is created by interdigital transducers placed in parallel or orthogonal arrangements to the microfluidic channel.

1.2.4 Single Cell Lysis

Single cell lysis or extraction of biomolecules from single cells can be achieved through various methods; the most efficient methods entail cells introduced to high-electrical fields, chemical lysis, and laser lysis as shown in *Figure 1.5*. Cells placed within high-electrical fields undergo electroporation leading to single cell lysis. Chemical lysis is achieved by the introduction of detergent solutions, facilitating the disruption of cell membranes. While laser

lysis is accomplished by implementation of a pulsed laser beam creating a cavitation bubble upon rupture of this cavitation bubble, shear stress is applied to lyse single cells [19,20].

Single Cell Lysis

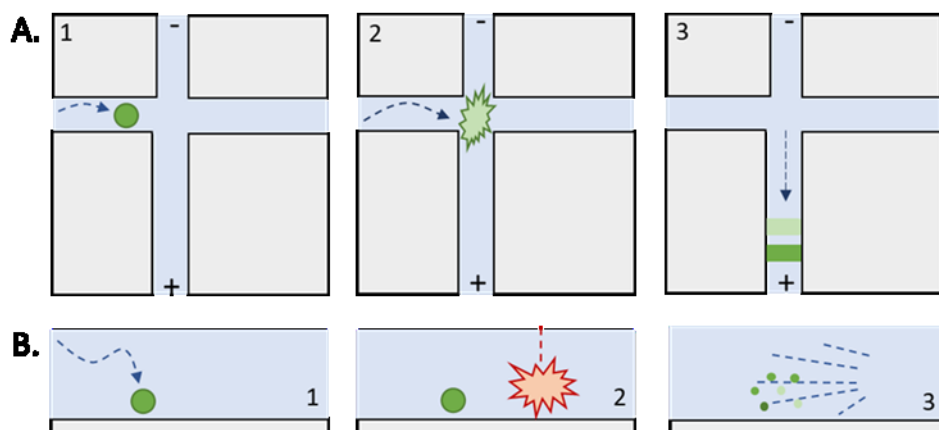


Figure 1.5 Single Cell Lysis Methods. A variety of single cell lysis methods exist, the methods with a higher visual appeal are (A) electrical lysis, whereby a cell enters an electrical field (1), the cell membrane is disrupted by the electric field (2), and the charged fluorescent biomolecules of interest are released for further interrogation (3). (B) Optical lysis is facilitated upon a cell (1) by the proximity-controlled creation of a picosecond laser pulse which produces a cavitation bubble (2). Upon the rupture and production of shear stress the biomolecules of interest are released (3).

1.2.5 Single Cell Detection

Single cell detection, specifically the ability to analyse the biomolecules liberated from single cells, can be achieved by a variety of microanalytical approaches as presented in *Figure 1.6*. The most prominent approaches for detection of biomolecules are fluorescent, amperometric and mass spectrometry detection. Fluorescent detection is a convenient and straightforward research application due to the abundance of commercialised fluorescent probes; such as fluorescent proteins, nanoparticles or quantum dots and specificity can be tailored to the biomolecule of interest. Detection of these fluorescent probes, in a microfluidic context, can be accomplished by laser-induced fluorescence (LIF). Amperometric detection depends upon integrating electrodes into a microfluidic channel allowing the detection of changes in ion content within the channel [20]. Another method that uses integrated electronic circuitry is a microcantilever sensor, a miniaturised diving board, by functionalizing this “diving board” with a selective layer towards the biomolecule of interest, biomolecules will be absorbed upon the surface creating stress and allowing content measurements. The ability to implement mass spectrometry detection for single cell analysis is limited due to the lack of technique sensitivity concerning the low number of proteins found in single cells. However, it is still highly desirable as the implementation of this method allows label-free quantitative

Single Cell Detection

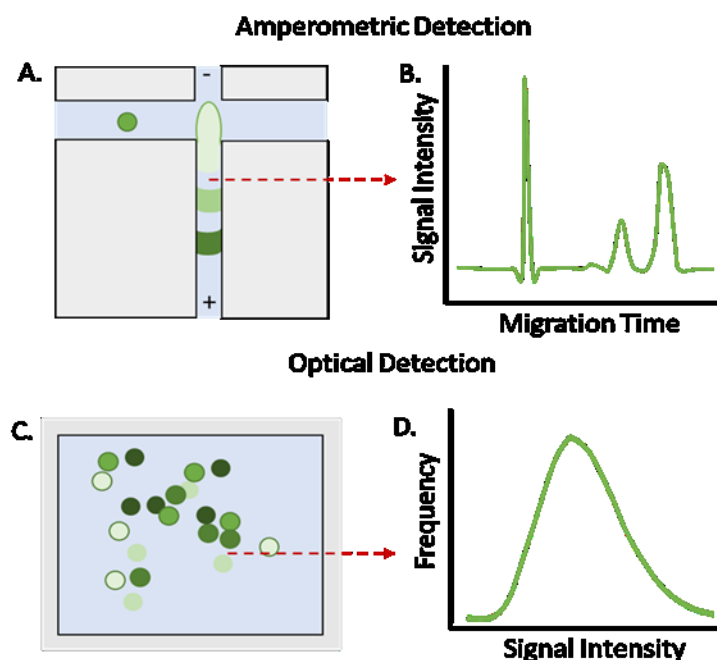


Figure 1.6 Single Cell Detection Methods. A variety of detection methods exist, (A) the ability to perform chemical cytometry through electrochemical or fluorescent detection of biomolecules is made possible by similar microfluidic device methodology of electrical lysis and induction of biomolecules. (B) The data generated from this detection method is in the form of single cell electropherograms. (C) Optical methods for fluorescent detection through the integration of fluorescent biomolecules produced within or fixed within organisms that reside within environmentally controlled microfluidic devices can be captured by epi-fluorescence microscopy. (D) Through the integration of computer analysis methods, the biomolecules of interest can be quantified.

analysis. Upstream separation has presented promising results towards combining microfluidic devices into this mass spectrometry detection system by implementing on-chip droplet or CE techniques.

1.3 Fluorescence

The ability to measure steady state or dynamic biological events, such as signalling, intracellular transport, and metabolism has become achievable, in part, due to developments in fluorescent probes and fluorescence instrumentation. Fluorescent probes are composed of a fluorophore, a compound which can re-emit light upon optical or electronic excitation, which can be coupled with a macromolecule (e.g., lipid, protein, nucleic acid, or synthetic polymer) [22,23]. The ideal fluorescent probe is engineered to contain a fluorophore with exquisite sensitivity coupled to a macromolecule with high selectivity. While the fluorescence instrumentation for detecting this generated fluorescence can be optimised through modifications of the excitation source, wavelength filters, and detection system.

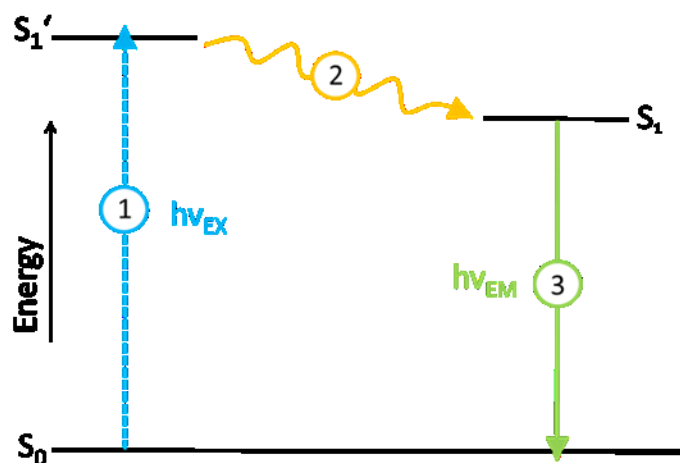


Figure 1.7 Fluorescence Process. The fluorescence process can be illustrated through the Jablonski diagram. (1) The creation of an excited electronic singlet state (S_1') in a fluorophore from the ground state (S_0) is achieved through energy absorption of an excitation photon ($h\nu_{EX}$). (2) this excited state exists for a finite time and relaxes into the singlet excited state (S_1) from which the fluorescence emission originates. (3) Upon fluorescence emission of a photon ($h\nu_{EM}$) the fluorophore returns to the ground state (S_0).

1.3.1 The Fluorescence Process

Generating fluorescence from a fluorophore is the result of a fundamental three-stage process [23], illustrated by a simple electronic-state diagram (Jablonski diagram) shown in *Figure 1.7*. The first stage of the process is excitation, where a photon of energy generated from an external excitation source, such as a laser or incandescent lamp, becomes absorbed by the fluorophore ($h\nu_{EX}$) creating an excited electronic singlet state (S_1'). This excited state, the second stage of the process, exists for a finite time (10^{-9} - 10^{-7} seconds), in which the fluorophore undergoes conformational changes and possible interactions with its molecular environment [24].

These processes result in two significant consequences, fluorescence emission (radiative decay) or competing processes (non-radiative decay). The fluorescence emission ($h\nu_{EM}$) occurs when the energy of (S_1') is partially dissipated yielding a relaxed singlet excited state (S_1). While competing processes may also depopulate (S_1'), such as chemical changes, collisional quenching and dissipation of energy as heat. A measurement of the relative efficiency to which these processes compete is descriptive of fluorescence quantum yield [25]. As presented in Equation 1.1, where quantum yield (ϕ) is a ratio of the photons emitted ($h\nu_{EM}$) to the number of absorbed photons ($h\nu_{EX}$).

$$\phi = \frac{h\nu_{EM}}{h\nu_{EX}} \quad (1.1)$$

This third step, the energy dissipation during the excited-state lifetime, returns the fluorophore to its ground state (S_0). Due to energy dissipation (by vibrating and rotating) during this excited-state lifetime, the energy of the photon emitted ($h\nu_{EM}$) is lower, causing the emitted light spectrum to be shifted to a higher wavelength (lower frequencies) [26]. This difference between the photon emission and photon absorption ($h\nu_{EX} - h\nu_{EM}$) is called the Stokes shift and is a fundamental concept towards the sensitivity of fluorescence techniques. As this allows the emission photons ($h\nu_{EM}$) to be detected against a background, which is isolated from the excitation photons ($h\nu_{EX}$).

1.3.2 The Fluorescence Spectra

As fluorophores are comprised of several vibrational levels within the electronic singlet state (S_1'), there is an increase in the number of possible absorption bands to such an extent that individual transitions cannot be resolved [25, 28]. Therefore, the resolved fluorophore's discrete electronic transitions, represented by $h\nu_{EX}$ and $h\nu_{EM}$, are in actuality, broad unresolved excitation and emission spectrums, respectively. These excitation and emission spectrums, as shown in *Figure 1.8*, supply information towards the wavelengths of light necessary to supply excitation and detect emission.

The excitation spectrum is acquired by measuring the emission intensity at one wavelength while the fluorophore is excited over a range of wavelengths. Over these range

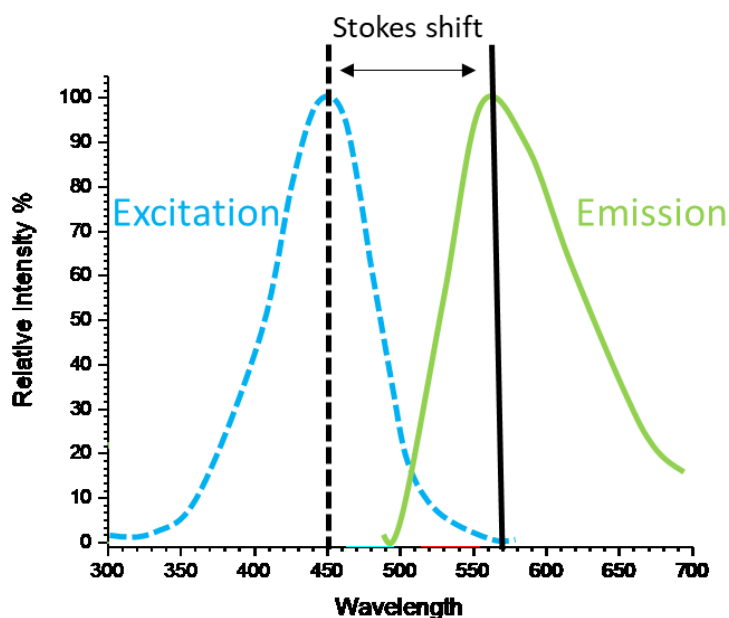


Figure 1.8 Fluorescence Spectra. Fluorophores can be characterized from their excitation profile ($h\nu_{EX}$) and the corresponding emission profile ($h\nu_{EM}$). The emission spectra are generated at longer wavelengths, due to the energy dissipation during the excited lifetime producing a lower energy in the photon emitted. The difference in energy or wavelength maxima (Excitation-Emission) is defined as Stokes shift. While there is a shift in the excitation to emission spectra, the emission spectrum mirrors that of the excitation spectrum.

of excitation wavelengths, there is no change in the profile of the emission spectrum. However, the emission intensity changes proportionally to the amplitude within the excitation spectrum. If no change occurs to the molecule in the excited state, then the excitation spectrum is identical to its absorption spectrum [28].

The absorption spectrum, a plot of absorbance concerning the wavelength of light applied, can be characterised by the implementation of a spectrophotometer. An instrument which transmits incident light (P_0) through a sample and measures the light power transmitted (P) through the sample [29]. The ratio of these measurements is defined as transmittance (T), as shown in Equation 1.2.

$$T = \frac{P}{P_0} \quad (1.2)$$

Most biological applications use absorbance (A), the amount of light absorbed by a solution, rather than transmittance (T). This measurement can be calculated through the logarithmic relationship as presented in Equation 1.3.

$$A = -\log(T) \quad (1.3)$$

The reason most biologists measure absorption rather than transmission, arises from the relationship defined by the Beer-Lambert law, which states the absorbance of a light absorbing solute is proportional to its concentration in solution [30]. This relationship is made possible through a solute's unique molar extinction coefficient (ϵ). This quantity can be measured, as shown in Equation 1.4, from dividing the absorbance (A) from the product of a known concentration of solute (C) and optical path length (d), usually defined by the width of the cuvette implemented.

$$\epsilon = \frac{A}{(d)(C)} \quad (1.4)$$

The emission spectrum is acquired by maintaining the excitation wavelength while the fluorophore's emission is measured over a range of wavelengths. As previously mentioned, the emission profile does not change by changing the excitation wavelength only the emission intensity will change. The emission spectrum occurs at wavelengths longer than the excitation wavelength, defined as Stokes shift [26]. Furthermore, the lower energy excitation spectrum is a predictable mirror image for the emission spectrum. Through

understanding a fluorophores characteristic of excitation and emission the correct wavelength can be applied providing peak amplitude excitation and in return allowing peak amplitude emission for detection.

1.3.3 Fluorescence Signal Detection

Regardless of the application, there are four critical elements for optimising fluorescence detection. These elements are the excitation source, the choice of the fluorophore, wavelength filters to isolate the emission photons from the excitation photons, and a detector that registers the emission photons [31]. The detector may produce a recordable output such as an electrical signal or photographic images.

The implementation of these four elements allows the creation of traditional fluorescence instrumentation, which primarily can be summarised into four different categories. The first category measures the average properties of bulk (μL to mL) samples and is classified as spectrophotometers and microplate readers [32]. Fluorescence microscopes are another category, which resolves fluorescence as a function of spatial coordinates in two or three dimensions for microscopic objects [33]. Flow cytometers are a category in high-throughput instrumentation for measuring the fluorescence per cell in a flowing stream, allowing the identification of subpopulations within larger cell samples[34]. While the last category, fluorescence scanners, allows spatial coordinate measurements of fluorescence upon macroscopic objects such as microarray plates, electrophoresis gels, blots, and chromatograms [35].

While instrumentation for fluorescence signal detection is various, it is important to note that each system makes different demands for the choice of fluorescent probe. Furthermore, each instrument also suffers from the production of different measurement artifacts. These limitations continue to promote the emergence of new technologies and improvements upon the traditional instrumentation. As evident through the fabrication of novel capillary electrophoresis apparatuses, DNA sequencers, and microfluidic devices to overcome demands and reduce fluorescence artifacts [31]. For example, the degradation of fluorophores (photobleaching) may impede accurate measurements through fluorescence microscopy, yet to overcome this challenge the creation of microfluidic devices which operate as flow cytometers, avoid prolonged exposure time to the fluorescence excitation beam, overcoming photobleaching limitations.

1.3.4 Quantitative Fluorescence

The fluorescence intensity of the distribution of fluorophores in a sample can be quantitative, that is the higher the fluorescence signal, the more fluorophores present [36]. This is due, in part, to fluorescence intensity being quantitatively dependent upon the same parameters as absorbance, as defined by Beer-Lamberts law, expressed in an unmodified format in Equation 1.5 with the absorbance (A) also expressed as the logarithmic ratio of the power of incident light (P_0) with the power transmitted through the sample (P) [30].

$$A = \varepsilon(d)C = \log \frac{P_0}{P} \quad (1.5)$$

The measured fluorescence intensity of the fluorophores present is representative of the power of the emitted fluorescence (P_F) from a sample. As shown in equation 1.6, this is equal to the amount of light absorbed by the fluorescent sample ($P_0 - P$) multiplied by the measured quantum yield (ϕ), previously presented in Equation 1.1.

$$P_F = \phi (P_0 - P) \quad (1.6)$$

If we substitute the explanation of absorbance from the Beer-Lambert law for the light absorbance of the fluorescent sample, Equation 1.7A is produced.

$$P_F = \phi P_0 (1 - e^{-\varepsilon d C}) \quad (1.7 A)$$

$$P_F = \phi P_0 (\varepsilon d C) \quad (1.7 B)$$

While the exponential in Equation 1.7A can be expanded, into a complicated scenario. For general use a reduction of the equation by approximation can reduce this equation to simpler forms as shown by Equation 1.7B, for practical purposes when the first term is deemed significant at small values of ($\varepsilon d C$), presenting the proportionality of emitted fluorescence (P_F) to the number of fluorophores present within a solution [37].

1.3.5 Factors Affecting Accuracy in Quantitative Fluorescence

While the previous theoretical discussion presents evidence for the emitted fluorescence (P_F) being directly proportional to the number of fluorophores present, there are other factors which affect this quantitative accuracy [38]. The first factor is that the proportional relationship between light absorption and fluorescence emission is only valid for cases in which the values within the Beer-Lambert law are small ($\epsilon d C$). Therefore, as the concentration of fluorophore increases, deviations present a non-linear association. Another characteristic distorting the linear relationship is fluorescence or concentration quenching. Arising when there is a non-radiative transfer of energy (S_1-S_0) between fluorophores, which can be pronounced at high fluorophore concentration [39]. While this spatial limitation in fluorophores affects quantitative microscopy, this energy transfer is exploited in another technique, called fluorescence resonance energy transfer (FRET). This technique allows the monitoring of biological interactions when biomolecules are in close proximity [40]. Through the non-radiative transference from a donor fluorophore (conjugated to a biomolecule of interest) to the acceptor fluorophore (conjugated to a different biomolecule) leading to fluorescence quenching in the donor and an increase in the acceptor's fluorescence.

Fluorescence intensity can also be impacted by high-intensity illumination conditions for sample imaging, leading to the fluorophores, under illumination, permanently losing the ability to express fluorescence (irreversible destruction) or a temporary loss of the ability to emit (photobleaching) [41]. These bleaching processes within the fluorophore can be explained by multiple photochemical reaction pathways, yet in all cases, the fluorophore modification originates from the triplet excited state, which is created from the singlet state (S_1), via the excited-state process called intersystem crossing [24].

Other factors influencing the day to day quantitative measurement capabilities of fluorophores are the pH of the solution and the temperature of the solution. It has been shown shifts in pH will sometimes radically affect the spectral characteristics and the intensity of fluorescence. This sensitivity is due to fluorophores being sensitive to changes in oxidative status (i.e., the presence of trace oxidising reagents) and the hydrogen network within the microenvironment of the chromophore [42]. This natural sensitivity, of some fluorophores to pH, has allowed the creation of novel intrinsic sensors, which when genetically encoded reveal intracellular pH measurements. The other factor, temperature, affects the viscosity of the medium, leading to an alteration in the number of collisions between molecules of the fluorophore with solvent molecules [36]. This day to day alteration

can be avoided by working at room temperature with solutions that are at ambient temperature before measurement.

Background signals, arising from the endogenous constituents in a sample (autofluorescence background) or non-specifically bound probes (reagent background) can severely compromise quantitative fluorescence detection [37]. Due to the background adding to the signal of interest, since the measured intensity value is the signal plus the background, as shown in *Figure 1.9*. This addition of signal reduces the signal to noise ratio (SNR) and dynamic range of the fluorescence measurement technique. Therefore its subtraction is necessary for quantitative techniques to increase the accuracy of the measured fluorophore intensity and present accurate fluorophore concentrations. Overcoming background signal can be achieved through either narrowing the fluorescence detection bandwidth within the fluorophore emission profile or choosing fluorophores with a fluorescent emission signal in a region where autofluorescence signal is low [21].

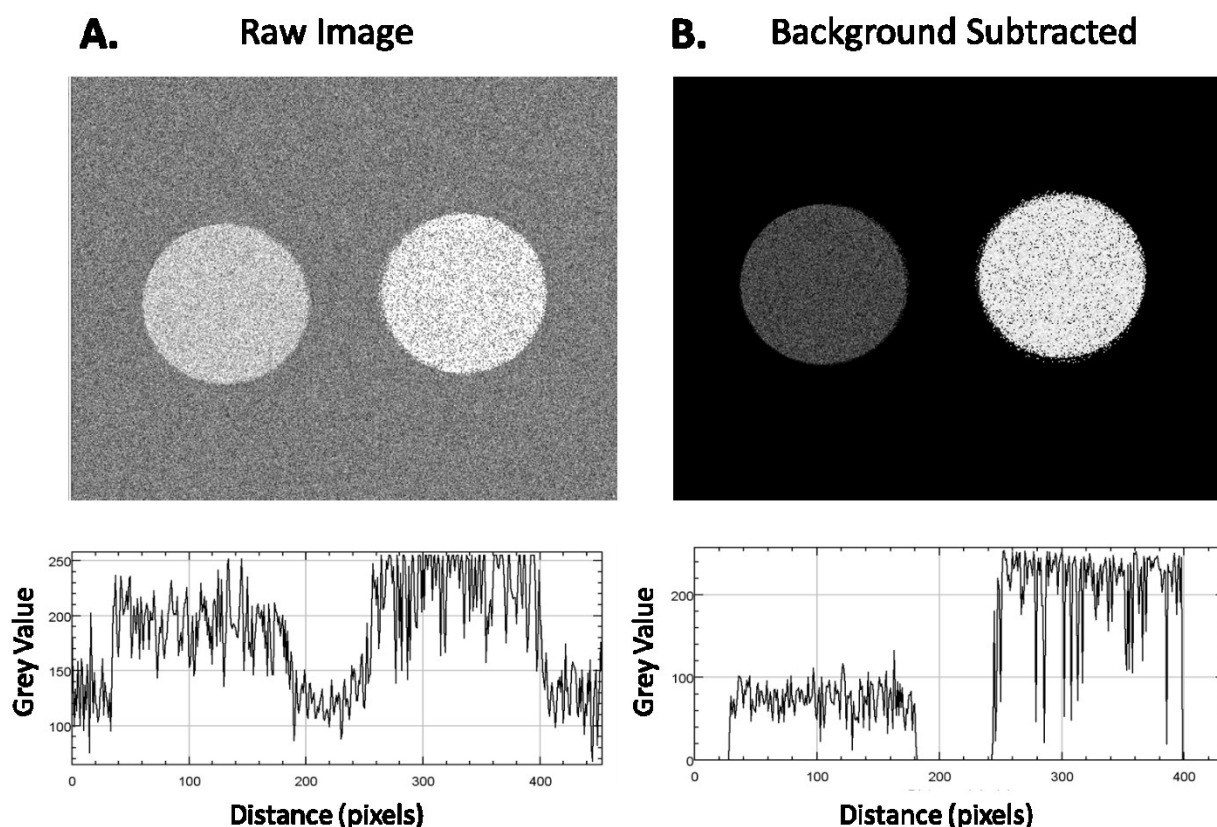


Figure 1.9 Fluorescence Background Subtraction. Any background in a measurement must be subtracted to provide accurate quantitation, this statement can be illustrated by (A) two circular cells in a raw image which contain a fluorophore of interest. The average grey value intensity of the cells is 195 and 223, left and right cell respectively. Without considering the background in the image it appears these two cells differ by 14%. (B) However, when the background is subtracted an average intensity of 70 and 218 is measured a 3-fold difference is noticed. This simple example shows the importance of background subtraction for accurate quantification of fluorescent intensities.

1.3.6 Enhancing the Fluorescent Signal

A straightforward way to enhance fluorescence signal is choosing a fluorophore with the highest fluorescence intensity (P_F), which can be estimated from the product of the molar extinction coefficient (ϵ) and quantum yield (ϕ) of the fluorophore [24]. Another way of increasing the measured signal is by increasing the number of fluorophores available for detection. However, this approach of simply increasing the local concentration of fluorophores can be counterproductive, due to the processes of fluorescence quenching. Another specific problem of increasing the number of fluorophores on a fluorescent probe is a decrease in the biomolecules specificity or binding affinity [43].

An alternative approach to increasing the fluorescent signal is through decreasing the fluorescent background achieved by implementing chemiluminescence, instead of fluorescence. While both processes yield photons (light production), fluorescence assays tend to have higher backgrounds compared to the exothermic chemical reactions applied to generate light from chemiluminescence compounds [44]. This little to no background interference allows the possibility of very low limits of detection.

This chemiluminescence phenomenon exists in nature and is called bioluminescence [45]. Firefly luciferase is one of the most commonly implemented bioluminescent reporters. The enzyme D-luciferin, a monomeric enzyme which facilitates this two-step process, emits light in the green to the yellow region. The first step is the activation of luciferin with ATP producing luciferyl-adenylate. The second step is the reaction of luciferyl-adenylate with oxygen to yield oxyluciferin, an electronically excited state. This excited state oxyluciferin then returns to the ground state through the release of light.

1.4 Proteomics

Proteins are the bridge between genotype and phenotype, understanding the abundance and interactions within the proteome presents answers to questions of biological function. In 1995, five years after the advent of the first μ TAS platform, the term “proteomics” was coined by Mark Wilkins and colleagues, a term defined as the characterisation of structure, function, and interactions of the proteins produced from a genome [46]. The central aim to global proteomics is not only to identify the proteins but also the interactions with other proteins, single nucleotide polymorphisms, functional aspects enhanced by post-translational modifications, and the localisation in perturbed and unperturbed conditions. A somewhat daunting task as the proteome is far more extensive than the genome due to alternative

splicing and protein modifications. Current proteomic studies, such as those in plant proteomics shown in *Figure 1.10*, are focused on two principal areas; functional proteomics, and expression proteomics [47]. Functional proteomics aims at describing the cellular mechanisms at the molecular level by probing the biological function of proteins such as protein-protein interactions. While expression proteomics aims at describing the quantitative and qualitative protein content, by exploring protein profiles in tissues which may alter due to growth conditions, stress, or cellular disease.

Through the quest to achieve this aim, many different research groups have risen with specified tasks, such as Functional Proteomics, Proteome Mining, Protein Expression Profiling, Post-translational Modifications, Protein-Protein Interactions and Structural Proteomics [47]. Through the collaboration of different research groups in Sweden and the UK, the lofty goal of protein localisation was achieved in 2017, concerning the human proteome. Where over 12,003 human proteins at a single-cell level were mapped to create the Cell Atlas, a framework within the far larger project, The Human Cell Atlas [48]. However, this has only been made possible by the emergence of new technologies and improvements upon the old ones which will be discussed in this section.

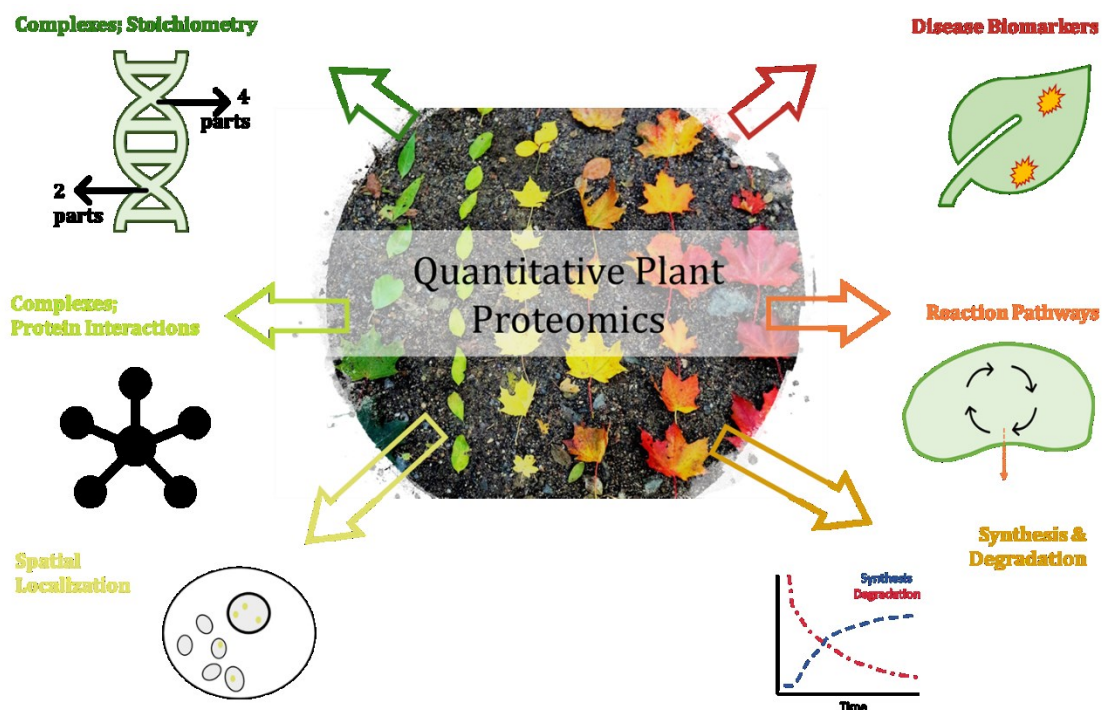


Figure 1.10 Areas of Quantitative Plant Proteomic Studies. Current approaches in both expression and functional proteomics have found a need for quantitative analysis. Such as determining the protein content with respect to spatial restrictions within the cell (spatial localization), the ability to identify disease from protein expression above or below a healthy threshold (disease biomarkers) with respect to increased /decreased synthesis or degradation rates. Through accurately measuring the stoichiometry or protein interactions (complexes), the biological modeling of these reaction pathways can.

1.4.1 Technologies for Proteomic Research

The techniques applied towards the analysis, identification, and quantification of proteins can be, for simplicity, separated into three steps 1) The preparation of the sample 2) separation of the protein mixture within the sample 3) and the characterization of proteins which have been separated or isolated from the sample [25, 26].

Creating the sample is frequently performed through cell lysis by harsh degradation methods inverting the millions of cell membranes present. Implementation of these methods can present beneficial aspects such as the ability to present a low salt content and still prevent contamination or degradation of proteins. Subcellular fractionation, whereby one uses equilibrium density centrifugation, differential centrifugation or density gradient centrifugation, to separate organelles and other subcellular structures for lysate creation allows higher specificity. Such that often proteins of interest, such as regulatory proteins are in low abundance and performing a pre-separation method increases the chance of achieving an identifiable readout.

A variety of separation techniques exist and can be divided into two categories either gel-based or gel-free methodologies [51]. A prominent gel-based methodology first applied towards proteomics in 1975, is two-dimensional protein electrophoresis (2-DE) allowing its implementation to separate proteins from *E. coli*, mouse and guinea pig [52], 2-DE consists of a first dimension, the isoelectric focusing, where proteins migrate in a gradient of acidity according to their intrinsic electrical charge. When proteins reach their isoelectric point, the pH at which a protein carries no net electrical charge, a band is formed. Upon the application

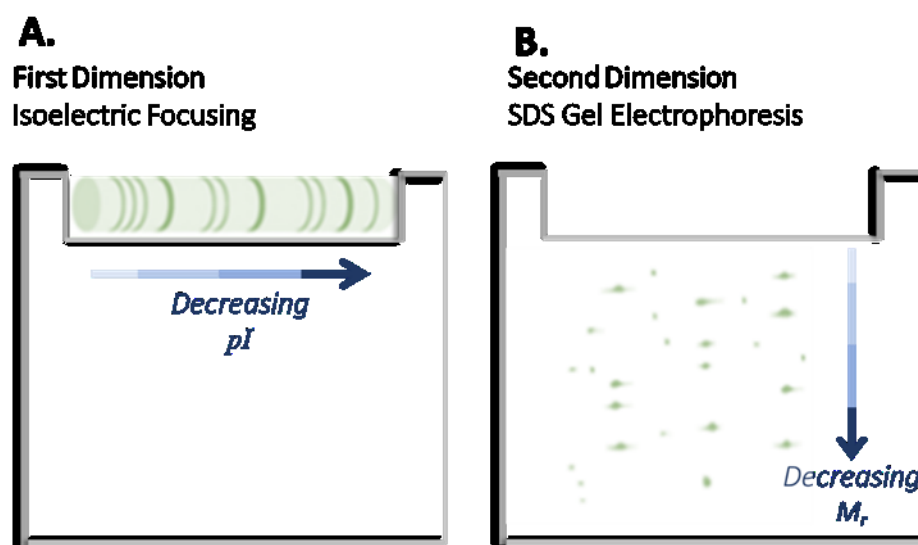


Figure 1.11 Two-Dimensional Gel Electrophoresis. A form of gel electrophoresis commonly used to analyze a mixture of proteins by two dimensions. (A) The first dimension involves separation of proteins by their isoelectric point (pI). (B) Followed by a second dimension of SDS-PAGE whereby proteins are separated by their weight (M_r).

of an electrical field the second dimension, SDS-Page is formed. Where proteins separate based upon their molecular weight into spots as shown in *Figure 1.11*. Variants of the technique of 2-DE have allowed Difference Gel Electrophoresis (DIGE) and three-dimensional protein electrophoresis (3-DE). While an alternative, prominent, gel-free method for protein separation is liquid chromatography (LC). This is performed by running a sample through an absorbing matrix, usually contained within a column. This matrix coupled with a desired eluting wash allows variation in protein separation by size, charge, or affinity for specific molecules or functional groups [49].

1.4.2 Protein Characterization

Visual demarcation coupled with a standard can achieve the characterisation of proteins using gel-based separation methodologies. Such that visual demarcation can be achieved by probing proteins with silver staining, Coomassie blue, or fluorescent demarcation through molecular probes, such as antibodies. Measurements can then be performed by gel-based densitometry.

The implementation of mass spectrometry may further characterise these visually demarcated spots. Mass spectrometry separates ions based on mass to charge ratio (m/z) and is a prominent method for the characterisation of proteins acquired by gel-free separation techniques, such as multidimensional protein identification technology (MudPIT) [53]. This consists of coupling both liquid chromatography with mass spectrometry. The preliminary implementation of mass spectrometry consists of reducing proteins into peptides by the addition of proteases or cleavage reagents. The peptides will undergo mass spec through three parts, the ion source to add a charge, a mass analyser, and a detection system to provide a peptide mass fingerprint representative of the proteins within the sample. While the implementation of databases such as MASCOT and SEAQUEST can identify these peptide mass fingerprints.

An alternative method for characterisation of proteins is protein microarrays or protein chips, where a solution of separated proteins become captured by high-density arrayed probes which are covalently linked to the surface of a silicon chip. Proteins can be visually demarcated by fluorescent molecular probes and by choice of the capture probe, as shown in *Figure 1.12*. This technique allows measurements of protein-protein interactions, protein-compound interactions and even relative expression levels [54].

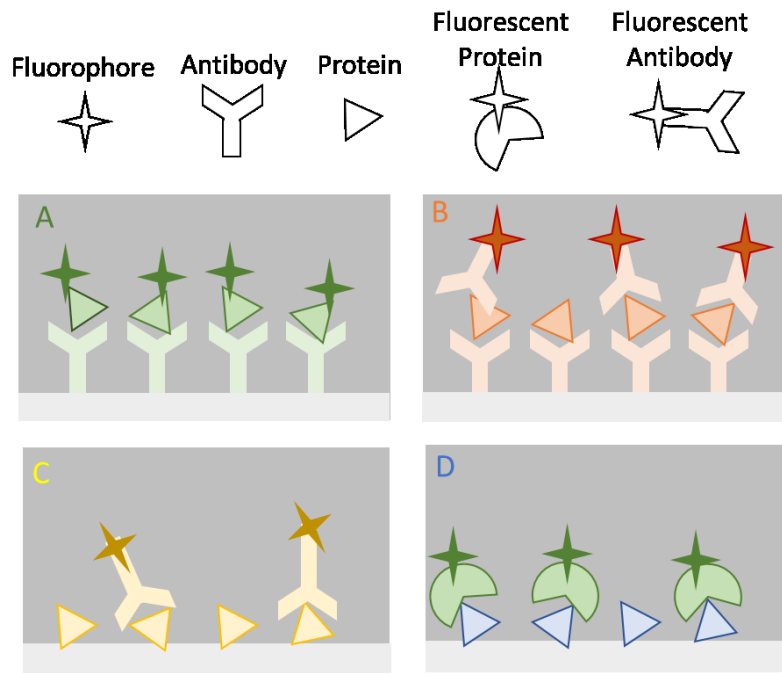


Figure 1.12 Protein Microarray Types. (A) label-based antibody array- where analyte is pretreated (conjugated or naturally fluorescent) to allow detection. (B) sandwich antibody array- where 2 antibodies are used to detect the analyte. (C) Reverse-phase array-where the analyte is immobilized on the surface. (D) Single-protein array-where only 1 protein of interest is used and immobilized on the surface.

1.4.3 Proteostasis

The proteomic content within a cell is under constant quality control, such that proteins are continually being synthesised, folded, trafficked, disaggregated and degraded. Proteostasis is the combination of these processes allowing regulation of the proteomic content within a cell [55]. The entry event towards these molecular processes is transcription. The process of transcription is initiated by transcription factors binding to DNA to call upon a convoy of RNA polymerase which transcribes the DNA nucleotide sequence into a pre-mRNA transcript. The pre-mRNA is processed through splicing of exons, capping, polyadenylation and final export from the nucleus, creating the mature form of mRNA. As the mRNA is composed of complementary base-pairing nucleotides and each group of three nucleotides constitutes a codon which specifies an amino acid, the sequential translation of these codons allows the synthesis of proteins as shown in *Figure 1.13* [56]. The rate of this translation is dependent on the rate at which ribosomes initiate translation, while the amount of translation is influenced by mRNA lifetime. This lifetime is influenced by multiple factors, such as miRNA binding, which adds further fine-tuning to gene expression [57,58].

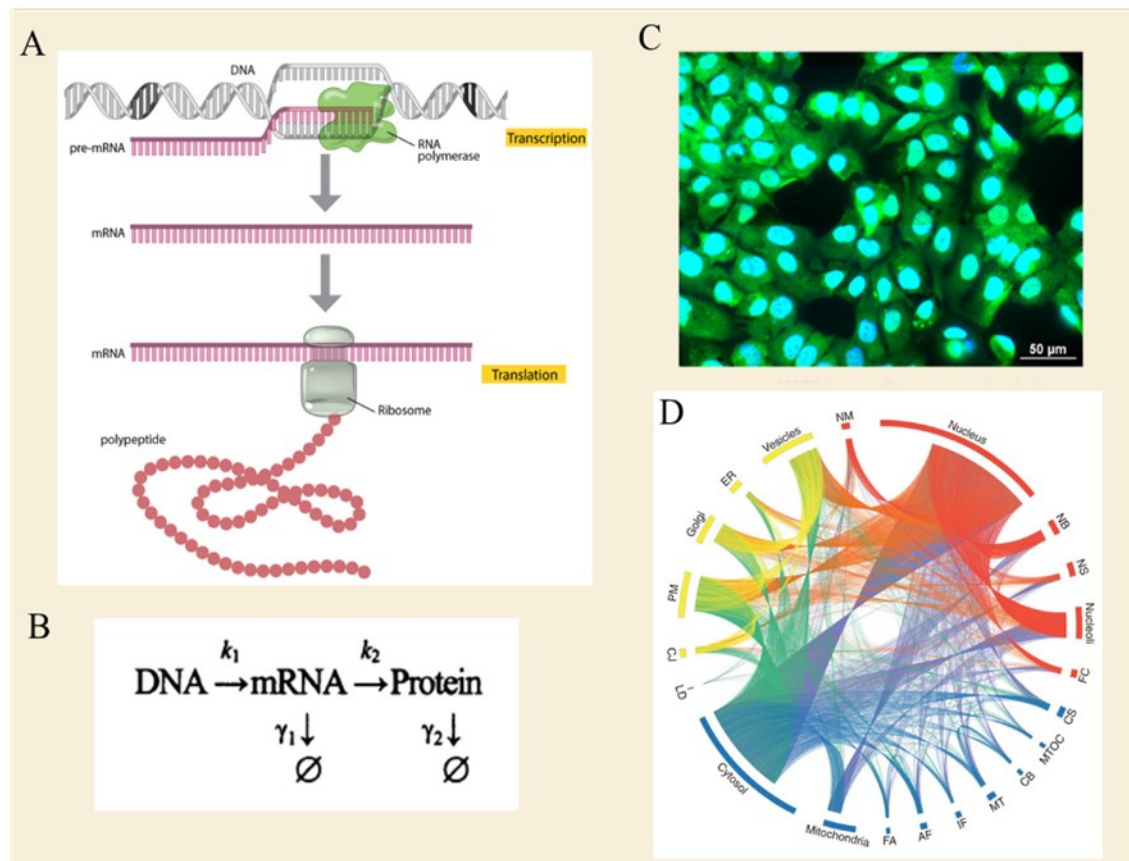


Figure 1.13 The Birth of a Protein: From Transcription to Translation Ending in Multi-localization. (A) Proteins are created, through the initial process of transcription, where a RNA polymerase (green) is procured to transcribe the gene encoded within the DNA producing a pre-mRNA transcript (pink). This pre-mRNA is processed to form a mature mRNA molecule which is translated by the Ribosome within the cytosol to create a polypeptide encoded by the original gene, adapted from [56]. (B) A two-stage gene expression model of the intrinsic noise generation through a proteins journey can be described by transcription rates (K_1) and translation rates (K_2) coupled with the rate of mRNA and protein degradation (γ_1 γ_2) respectively, adapted from [40]. Understanding the migration of proteins can be performed by multiple proteomic techniques, such as Immunofluorescence (IF) Microscopy (C) where proteins are fluorescently labeled through the anti-protein antibody coupled with a fluorescent marker, allowing spatial insight towards proteomic measurements. This approach of antibody-based image profiling has allowed localization data for 12,003 proteins as shown in (D) whereby locational acronyms are NM-Nuclear Membrane, CS-Centrosome, AF-Actin Filaments, PM-Plasma Membrane, NB-Nuclear Bodies, CB-Cytokinetic Bridge, FA-Focal Adhesions, NS-Nuclear Speckles, MT-Microtubules, LD-Lipid Droplets, FC-Fibrillar Center, IF-Intermediate Filaments, CJ-Cell Junction. Adapted from the Human Cell Atlas [48].

As proteins migrate from their primary to functional tertiary structure, facilitated by multiple unpredictable routes and intermediate conformations representative of an “energy landscape theory” [59]. Proteins are transported to multiple locations as shown in *Figure 1.13 D*, to facilitate interactions with small-molecule substrates (enzymatic or signal-cascades), other proteins (protein-protein interactions), other proteins within a protein complex (quaternary structure), and even with DNA (transcription factors) to create more protein “friends” to help carry out all the functions necessary for cellular processes. To maintain proteostasis, while new proteins are being synthesised older, misfolding or aggregated proteins are marked by ubiquitin. This ubiquitin-mediated post-translational modification targets protein for degradation to either the proteasome or lysosome [60].

1.5 Cell-to-Cell Variability in Protein Expression

The field of single cell biology is ripe with variations in; cell size, cell cycle position, protein content, and even the number of organelles in a single cell. This variation is inherent within living systems, due to the stochastic as in, sporadic in time, behaviour and low copy number of components regulating these cellular processes [61]. This produces single-cell variation and is responsible for a diverse biological phenomenon, the emergence of “outlier cells” which are crucial in the creation of antibiotic resistance [62], cancer stem cells [63], and alternate responses to viral infections [64]. As the proteomic components are responsible for the interactions giving rise to the observed phenotype, their accurate quantification at single-cell resolution is necessary and has been made possible through the emergence of single-cell analysis technologies. Furthering our understanding and ability to model the biological systems that give rise to this cellular heterogeneity [65].

1.5.1 Time-lapse vs Population Measurements

Single-cell analysis technologies acquire data by either population or time-lapse measurements. This latter measurement technique involves tracking individual cells over time, quite often through fluorescence microscopy techniques coupled with tracking algorithms allowing the reconstruction of each cells protein abundance over time [66]. Providing far more information, in the form of protein content evolution within a cell and future post-divisional generations, in comparison to population measurements. Where all single cells are measured only once, due to limitations in the data acquisition process. Occurring as either cells being measured in random sequential order (i.e. flow cytometry) or cells do not survive the acquisition process (i.e. fixing and staining).

While population measurements lack the temporal resolution of time-lapse measurements, it can be inferred that population distributions reflect solely temporal fluctuations within individual cells. This is based on the ergodic hypothesis which implies that the time average equals the ensemble average [67]. Meaning, multiple measurements of the temporally fluctuating protein within an individual cell is equal to one measurement of the protein content in multiple temporally fluctuating cells.

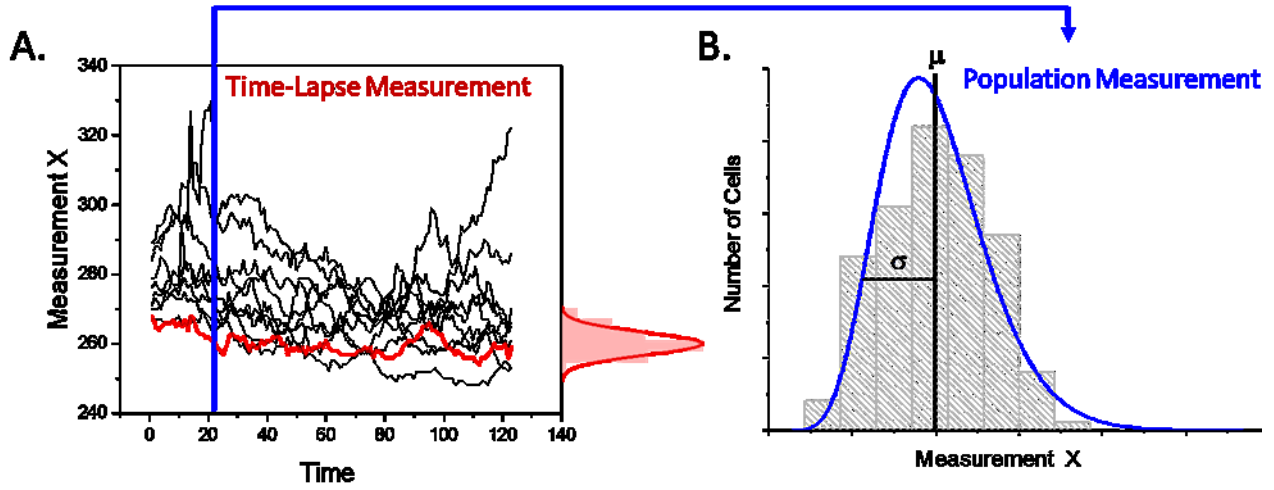


Figure 1.14 Single Cell Measurements. Two differing processes for single cell analysis exist. (A) The first class of data is acquired through time-lapse measurements, resulting in data which individual cells can be tracked over time. Such as tracking a measurement of X in a cell over a given time-course. (B) An alternative technique would be to measure multiple temporally fluctuating cells, which provides a population measurement. Through the basis of the ergodic hypothesis, which implies the time average equals the ensemble average, both techniques provide insight to the temporal fluctuations of measurement X in a cell.

1.5.2 Defining the Noise in Protein Expression

As discussed in section 1.4.3, the production and maintenance of proteins is a multistep process between transcription and translation, descriptive of the central dogma of molecular biology. With measurements of temporal resolution applied to this process, it has been shown the process is further complexed by the mechanism of bursting. A term for the creation of multiple products from one substrate. Two types of bursts, transcriptional bursts and translational bursts exist [68]. Respectively explained as many mRNA molecules produced from a single gene or many proteins produced from a single mRNA.

These two bursting events comprising protein expression are reliant upon two key parameters, the *burst size* (number of product molecules from one substrate) and the *burst frequency* (time between bursting events). When these parameters are randomly executed, with a uniform probability over time, the measured distribution will represent Poisson behaviour [69]. Deviation from this Poisson behaviour can be measured by the noise strength ($FF\phi$), also called the Fano Factor, which is the ratio between the variance (σ^2) and the mean (μ), as presented in Equation 1.8. For a Poisson distribution, the Fano Factor would equal 1.

$$FF\phi = \frac{\sigma^2}{\mu} \quad (1.8)$$

When these parameters are invariant and nonuniform (i.e. the burst size is large, and the burst frequency is seldom) the measured distribution will exhibit a larger Fano factor, therefore a higher variance in cell-to-cell measurements. The balance of these dynamic processes is reflected in the measured protein abundance or directly measured by real-time monitoring of protein production [70, 71].

This resulting protein product from these stochastic processes is a distributional measurement in which the variance, the cell-to-cell variability in the measure, is defined as biological noise [72]. Quantified by the squared coefficient of variation (η^2); that is, the variance (σ^2) of protein abundance normalised by the square mean (μ^2) as presented in Equation 1.9.

$$\eta^2 = \frac{\sigma^2}{\mu^2} \quad (1.9)$$

1.5.3 Intrinsic vs Extrinsic Noise in Protein Expression

While the coefficient of variation (η) descriptive of the random fluctuations (σ) around equilibrium (μ) is usually not significant in macroscopic chemical reactions (i.e. test-tube chemistry), as the number of molecules present is so numerous their stochastic fluctuations average out. This behaviour is rooted in the finite number effect combined with the central limit theorem and can be explained by Equation 1.10.

$$\eta^2 = \frac{1}{\mu} \quad (1.10)$$

This highlights the fundamental relationship between system size and noise, that is noise tends to increase when the size of the system is decreased [73]. Consider a protein moving freely between two systems, the smaller chloroplast and the larger cytoplasm. If the systems are at equilibrium, with respect to concentration and one protein moves from the 1,000 proteins present in the cytoplasm into chloroplast which contains 10 proteins, there is a 10% change in the chloroplast concentration, but only a 0.1% change in the cytoplasmic concentration. This differential effect arising from the different number of molecules within a system is the finite-number effect and is a dominating effect in the creation of molecular-level noise in cellular regulation.

Furthermore, noise (η^2) is a summation of intrinsic (η_i^2) and extrinsic (η_e^2) noise sources [74]. These sources can be defined respectively as noise generated directly with a given process and noise generated from environmental factors associated with the process and can be calculated from Equation 1.11.

$$\eta^2 = \eta_i^2 + \eta_e^2 \quad (1.11)$$

While both noise sources are influenced by the finite number effect, intrinsic noise usually refers to the stochastic variations involved directly within the production of a gene product (i.e. transcription, translation). In contrast, the extrinsic noise arises from factors that do not directly participate in gene expression, such as cell cycle effects, upstream network contributions, and alterations in environmental factors [62]. While extrinsic noise factors are not direct participants, they do affect gene expression in a non-trivial manner. Recent evidence through molecular modelling provides insight that the addition of extrinsic noise produces distributions with a greater variance to mean relationship than what would be expected by a Poisson distribution ($FF\phi=1$) [75].

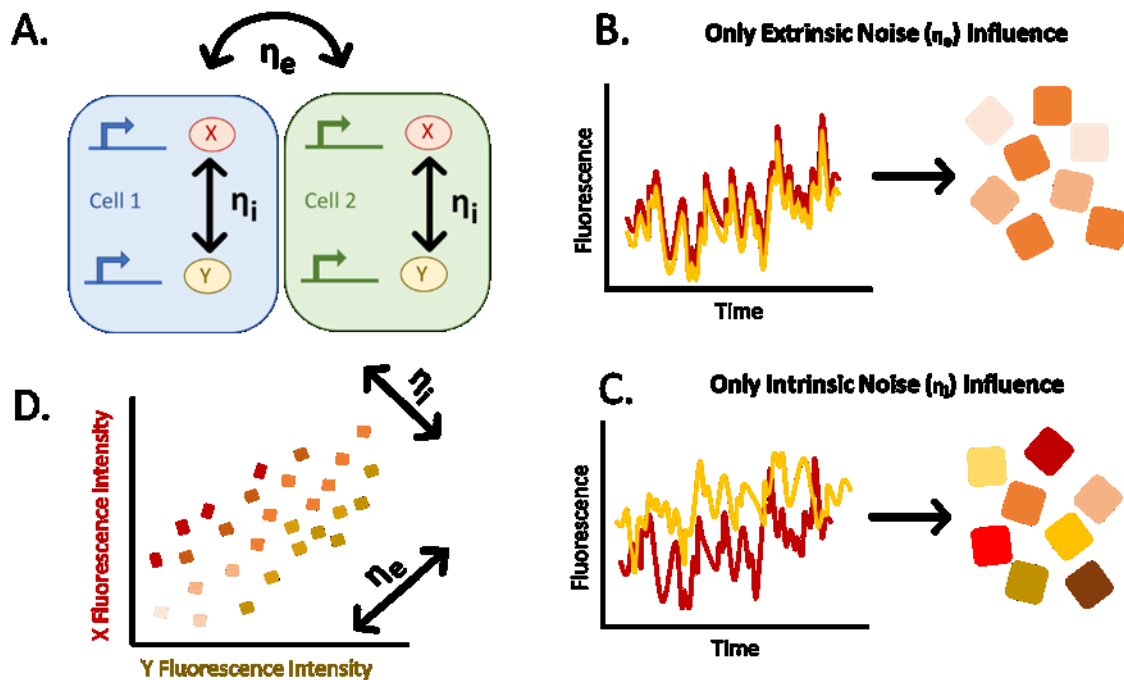


Figure 1.15 Measuring Intrinsic and Extrinsic Noise. The ability to measure extrinsic (η_e) and intrinsic (η_i) noise is provided through (A) an experimental setup in which two fluorescent proteins (X and Y) under identical regulatory sequences are manufactured in a cell, allowing measurements of intrinsic noise. While the differences between the cellular environments allows a measurement of the extrinsic noise. (B) If no intrinsic noise was present, the extrinsic noise would influence cells to contain different intensities but the same mixture of a fluorescent color. (C) If no extrinsic noise was present, the intrinsic noise would influence cells to produce a different mixture of fluorescent color. (D) Through plotting each cell's fluorescent intensity for both X and Y the variation of intrinsic and extrinsic noise within the total distribution can be quantified.

It is the combined effect of both the extrinsic and intrinsic noise that leads to phenotypic variations and current single-cell analysis techniques have presented this cell-to-cell variability in protein expression to be a gamma distribution [76]. This statistical distribution arises naturally concerning the waiting times between Poissonian distributed events [77]. Where the Poissonian distribution allows a prediction of the number of events (bursting) occurring ≤ 0 times in an interval. Descriptive of the probability of observing (k) events with an average number of events (μ) expected in time.

$$P(x = k) = e^{-\mu} \frac{\mu^k}{k!} \quad (1.12)$$

While the gamma distribution describes the probability of observing a protein copy number in a single cell ($p(x)$) in relation to the shape parameter (α) and the scale parameter (β). These parameters can be associated with the waiting times (burst size and frequency) between the biochemical events that compose protein production [71].

$$P(x) = \frac{x^{\alpha-1} e^{-x/\beta}}{\Gamma(\alpha) \beta^\alpha} \quad (1.13)$$

1.6 Single Cell Protein Analysis

Over the past decades, analytical techniques have progressed, through a variety of hybrid methods produced by collaborative cross-fertilisation, transforming the microfluidic and non-microfluidic tools available to understand the heterogeneity in cell populations. These methods have provided single cell resolution measurements of physical properties, cell signalling, DNA/RNA sequencing, and proteomic analyses [78]. As to not be exhaustive, the focus shall be on the latter by presenting a selection of exciting and impactful techniques similar to the proteomic measurements acquired by the MAC chip.

Understanding the phenotypic impact of cell population heterogeneity has allowed further progress in a variety of contexts; such as personalised medicine, uncovering highly specialised cells that present distinguishing features in plants and the phenotypic consequence of bacteria response in the presence of antibiotics [79, 80]. Nevertheless, even with these advances, not one analytical method can provide quantification of all the protein

parameters in a single cell. Hence, why a variety of biochemical and biophysical methods continue to be developed and improved.

1.6.1 Single Cell Fluorescence Microscopy

Fluorescent-based methods for protein detection, combined with epi-fluorescent microscopy, and with state-of-the-art image processing has been proven to be a powerful tool for time-lapsed, high-throughput measurements of live single-cell protein abundance [81, 82]. The field of proteomics measurements by fluorescence microscopy has undergone a vast transformation with the creation of novel imaging methods such as fluorescence resonance energy transfer (FRET), Photo-activated localisation microscopy (PALM), fluorescence lifetime image microscopy (FLIM), and stochastic optical reconstruction microscopy (STORM) [83]. Furthermore, the ability to integrate single molecule resolution coupled with small microfluidic volumes is driving relative improvements towards the detection limit

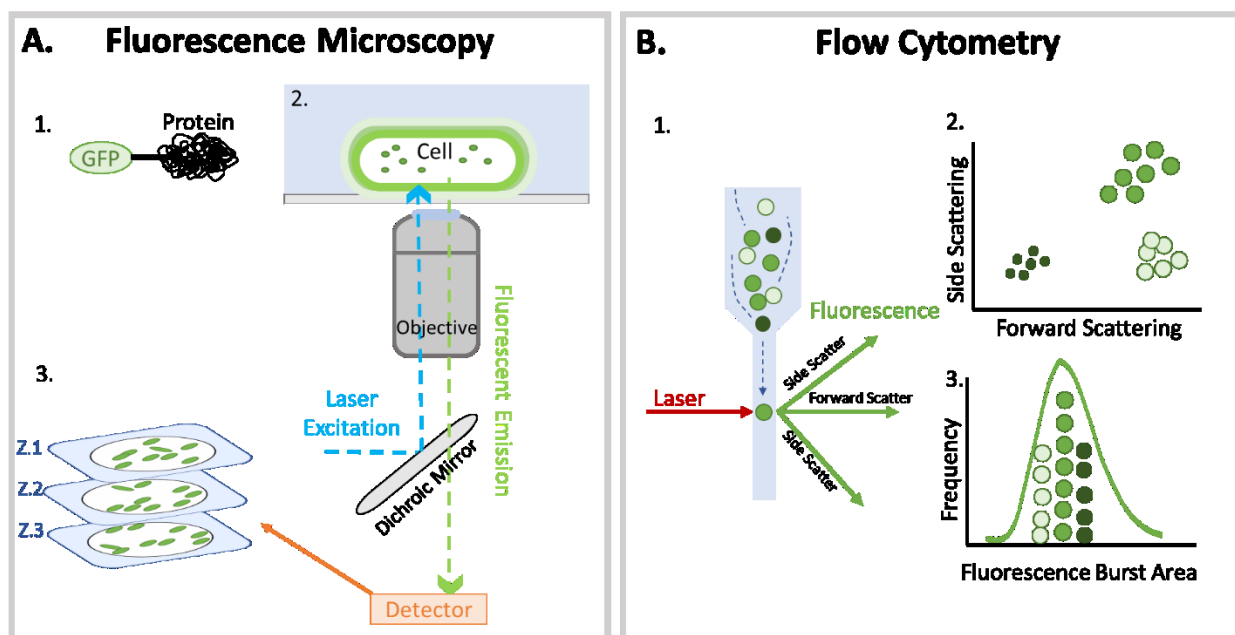


Figure 1.16 μ Fluidic Fluorescence Microscopy and Flow Cytometry Single Cell Protein Analysis. (A) Fluorescence microscopy combined with fluorophore-fusion libraries have revolutionized the field of quantitative protein analysis. (1) fluorophore fusion libraries are created by fusing a fluorophore of interest to the C-terminus of a specific protein, in its native chromosomal position, producing strains with respect to the protein of interest. (2) The genetically modified organisms can then be flushed into microfluidic lanes (respective of strain) and illuminated through fluorescence microscopy. The proteins of interest within the cell are excited by a laser, which passes through the objective, and the emission photons are captured by a detection source. As the dichroic mirror exhibits different reflection and transmission wavelength properties, the same objective can be implemented for both excitation and emission. (3) The images acquired resolve the fluorescence as a function of spatial coordinates in two dimensions or three dimensions, the latter requires image stacking in the z-dimension. (B) Flow cytometry methodology. (1) entails using spatial limitation, so that single cells can be analyzed while passing through a laser source. Multiple detectors quantify both the intensity of the fluorophore emission, side scatter (cellular granularity), and forward scatter (cell size). (2) These multiple measurements make flow cytometry a powerful tool for protein quantitation with respect to cell size and granularity, allowing the possible identification of sub-populations or noise isolation from extrinsic noise factors. (3) Distribution profiles of fluorescence intensity (fluorescence burst area) can provide quantitative proteomic measurements when the fluorophore to protein ratio is 1:1 and the platform has been calibrated so that a fluorescence intensity corresponds with a known number of fluorophores.

during this new era of “digital biology” [84]. Such that through coupling the complete *E. coli* genome with YFP the creation of fluorescent protein fusions (FPF's) allows single molecule quantification of the proteome [85]. This technology has allowed researchers to evaluate the variability in protein expression levels in bacteria, uncovering the statistical bounds of protein expression in bacteria which has been shown to be well described by a gamma distribution.

Through further work, upon mammalian cells, it has been proven that biological noise, representative of the cell-to-cell variability fits a gamma distribution by both theory and experimental studies [71]. Quantification of the attributed sources upon this measured cell-to-cell variability has been made possible through the implementation of fluorescent protein expression under identical regulatory sequences measured by fluorescence microscopy [62]. This technique provides insight towards the biochemical meaning of the gamma distribution parameters while separating the total noise measured into extrinsic and intrinsic noise sources. Intrinsic noise sources are defined as the random molecular birth and death process within protein creation. Extrinsic noise sources are identified as the variations of regulatory molecules, signalling cascade components, and fluctuations of cytoplasmic and nuclear volume [86].

To examine this cell-to-cell variability in protein expression during leaf maturation, Araújo et al. have measured the photoconvertible KikGR marker under expression control of two constitutive promoters, allowing insight into the intrinsic and “dominant” extrinsic noise during leaf development [87]. While the innovation of fluorescent probes has led to single cell analysis breakthroughs in fluorescent microscopy another high-throughput methodology, flow cytometry has also received equal benefit.

1.6.2 Single Cell Flow Cytometry

In comparison to fluorescence microscopy, flow cytometry derives similar effectiveness for measuring small absolute protein amounts per cell as this method allows cell intactness to be maintained creating localised protein concentrations. Flow cytometry was a critical approach towards uncovering the intrinsic noise for cell-to-cell variation in the expression of the GFP-tagged proteome in *S. cerevisiae* [88]. Through gating, defined as reducing the forward and side scattering gate radii to negate cell size differences, one can measure the significant contribution of intrinsic noise. As represented as the unique inverse-proportional relationship between the coefficient of variation (CV^2) and protein abundance. Ungated

populations present measurements of total noise within the cell-to-cell variation exhibiting an extrinsic noise dominance [89].

While coupling fluorescent proteins to the entire proteome, may provide comparative protein copy number measurements, information upon the phosphorylation status of these proteins is lost. Also, the coupling of one fluorescent indicator causes the inability to perform multiplexed measurements upon significant protein-protein interactions. However, with the advent of multicolour flow cytometry in the early 2000's both the Roederer and Nolan group, presented the ability to measure 10-15 proteins in signalling pathways. Furthermore, post-translational modification status upon these pathway kinases was achieved by the ability to fix and stain cells with probes that target phosphorylation status [90]. Also, through integrating downstream cell sorting applications with flow cytometry, such as fluorescence-activated cell sorting, it has become possible to interrogate protein distributions for specific cell-type populations. Through methods as different GFP-tagged cell-types in the Arabidopsis root apex or even labelling the nucleus of cancer stem cells with Hoechst 3342 [91, 92].

A drawback to commercial flow cytometers is the requirement of a large number of cells. Therefore it is beneficial to integrate microfluidics as flow cytometers, allowing a decrease in the number of cells to 100-1000 as the volumes for sample preparation is reduced. This decrease in volume allows absolute quantitation of intracellular protein content, comparative to commercial flow cytometry methods, due to constriction of the area in the microfluidic channel manifolds [93]. Furthermore, this reduction in volume has allowed single mitochondria absolute protein measurements on the proteins Bcl-2 and Bax, proteins involved in the process of cell death. Measurements are acquired by extrapolating the number of fluorescently conjugated antibodies to the proteins of interest after calibrating this highly sensitive flow cytometer by FluoSpheres (Fluorescent nanoparticles). These measurements present that heterogeneity not only exists at the level of single cells but also within the mitochondria [94].

1.6.3 Single Cell Western Blotting

While absolute quantification upon a single intracellular protein in flow cytometry is exceptionally beneficial towards bettering the current biological models, reasoned by bulk measurements resulting in single cell estimates for protein content which are 3-10 times higher than those in single-cell experimental studies [95]. Further advancements would be possible through the ability to perform not only quantitative but multiplexed single-cell measurements. Through the creation of miniaturised alternatives to western blots based on

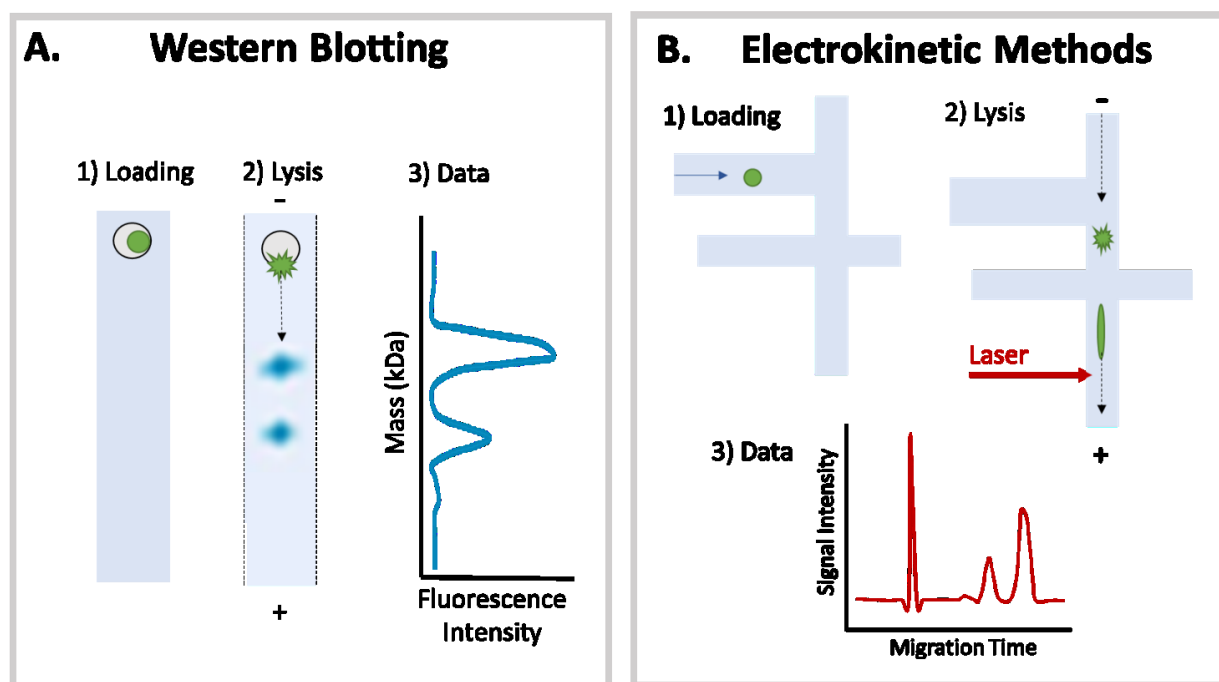


Figure 1.17 μ Fluidic Western Blotting and Electrokinetic Single Cell Protein Analysis. (A) Western blotting of bulk protein suspensions is a traditional approach for quantitative proteomic studies. The creation of single cell western blotting implements the technologies of SDS gel electrophoresis, yet in a miniaturized format (1) so that single cells are loaded into microwells (2) and undergo electrical lysis. (3) The data generated is akin to that from bulk western blotting methods, where proteins migrate based upon their weight, allowing mass identification coupled with quantitation from signal intensity. (B) Electrokinetic methods such as CE in a microfluidic format allow both (2) single cell lysis and (3) biomolecule detection in one microfluidic channel. The lysate can be analyzed through laser induced fluorescence and biomolecules exhibit migration times, based upon the charge of the biomolecule.

microfluidic or CE, achievements have been accomplished towards producing high-throughput, automation, and higher mass sensitivity [96]. Such as shown by the Herr group through the creation of single cell western blotting. This technique allows multiplexed protein measurements (11 proteins per cell) with a quantitative resolution of <30,000 molecules [97]. Furthermore, the Herr group has progressed towards bi-directional electrophoresis within single cell western blotting, allowing the measurements of both F-actin and G-actin heterogeneities in populations of MDA-MB-231 GFP-actin cells [98].

1.6.4 Single Cell Electrokinetic Methods

The integration of electrokinetic methods into microfluidic devices has presented multiple advantages through both the concentration and separation of samples [4]. Isotachopheresis (ITP), whereby a sample is placed between two electrolytic solutions of differing ion mobility, is an ideal preconcentration method and has been applied to a variety of protein immunoassays increasing their sensitivity to detect low concentration protein species [99,100]. Through the fabrication of microfluidic channel junctions, such as T or cross-junctions, coupled with an electric field allows one the ability to perform electrokinetic injection of single-cell lysate or further analysis, as shown in *Figure 1.17 B* [101]. As the cell

lysate undergoes CE the targeted proteins of choice undergo separation and LIF detection to measure innately fluorescent proteins or proteins tagged with a fluorescent antibody. This measurement technique allows single-cell electropherograms, as shown in *Figure 1.17B 3*, which quantify fluorescent protein content by integrated fluorescence [102,103]. When these measurements are transformed into digital readouts by implementing single-molecule fluorescence counting these electropherograms maintain the ability to measure lower copy number proteins in single cells (1×10^3) [104].

1.6.5 Single Cell Affinity Arrays

The possibility of measuring secreted proteins (the secretome) has become actualised by the combination of microfluidics and enzyme-linked immunosorbent assays (ELISA), as demonstrated in *Figure 1.18*. The microfluidic aspect provides small volumes through the integration of valve structures or the creation of microwells. While the ELISA methodology is facilitated by multiple strips of antibodies capable of capturing specific proteins. By coupling these protein barcodes with microarray scanners measurements provide medium-throughput data analysis capabilities when average fluorescent signal rather than digital

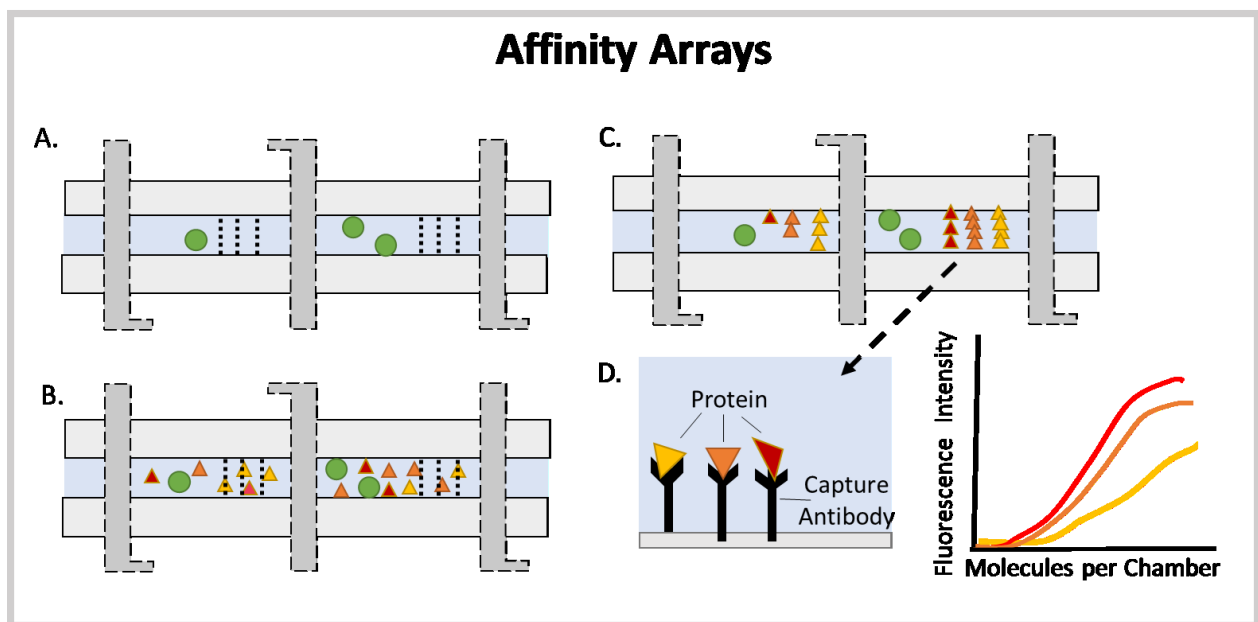


Figure 1.18 μ Fluidic Affinity Arrays for Single Cell Protein Analysis. Scaling down protein characterization technology such as protein microarrays, displayed in Figure 1.12, and implementing them within a microfluidic environment can be advantageous for single cell biology. (A) Single cell resolution is achieved through the integration of microfluidic valving to isolate cells into small chambers containing barcodes (black dash line). The barcodes are fabricated through immobilization of a protein capture agent. (B) While lysis methods can be applied, lysis is not necessary to assess a cells secretome. Over time cells secrete proteins of interest (C) the capture agent binds to these secreted proteins creating barcodes. (D) After the calibration of the fluorescence intensity of the barcodes with respect to the number of molecules in the chamber, quantitative secretome analysis can be actualized.

single molecule signal is necessary to quantify protein content. The creation of microfluidic microwells allows the user an ability to trap ~1 cell per well, which upon cell incubation with antibodies specific towards secreted proteins, such as IL-6 and IFN, measurements upon the secretome can be achieved [105].

Heath's group actualised this technique into a microfluidic device titled the single-cell barcode chip (SCBC) which allows the assessment of 12-proteins showing secretome heterogeneity in phenotypically similar T cells [106]. This technology was also applied to secretion profiling of IL-8 and VEGF proteins from circulating tumour cells, isolated from patient blood samples [107]. Through the adaptation of lysis buffer into the SCBC further absolute quantification of 11 cytoplasmic and membrane proteins involved in the EGF-receptor mediated PI3K signalling pathway is achieved [108].

1.7 Microfluidic Platforms for Plant Cell Analysis

The interdisciplinary science between plants and microfluidics in recent years has been limited towards the studies of whole (living) organisms, as shown in *Figure 1.19*, allowing biological insight towards pollen tubes, cotyledon growth, hyphae, and roots [69,70]. The choice of implementing microfluidic technology compared to conventional methodologies presents multiple advantages such as; shorter assay times, higher resolution, and sensitivity at the microscale while allowing less sample and reagent consumption. Presenting the beneficial argument for the continued implementation of microfluidic technology to meet research demands in plant biology.

1.7.1 Towards Single Plant Cell Analysis

Recently, it has been recognised that single-cell experimental outputs may provide a different perspective for plant biology, possibly revealing the plasticity, developmental trajectory, and responses at a single-cell level towards environmental inputs [79]. Indicating a shift of experimental approaches from pooled cell profiling to single-cell profiling. Through the development of novel microfabricated tools or tailoring the existing ones for plant biology research, it is possible to meet these experimental needs.

Previous research into the integration of plant cells into a microfluidic device have presented the ability of growing an entire plant in a microfluidic environment [111, 112]. While these devices show the ability to maintain non-dedifferentiated cells, these techniques still pose a significant challenge for single cell interrogation. It has been demonstrated that

single-cells within an organism can be interrogated by extracting the protoplasm through microcapillaries [113]. However, to perform measurements upon a more extensive population of cells residing in internal tissues the most widely accepted method is through enzymatic cell wall digestion [114]. Using this approach, one can obtain single mesophyll protoplasts which have been shown to maintain their photosynthetic capability for over nine hours [81]. Allowing microfluidic single plant cell experiments to be performed, yet only to culture and measure division events of protoplasts after dedifferentiation, losing valuable information concerning the cells previous specialised function [115, 116]. This information loss leads to a significant gap in single plant cell research which can be answered by a platform capable of harbouring single plant cells before dedifferentiation with an integrated single-cell measurement system.

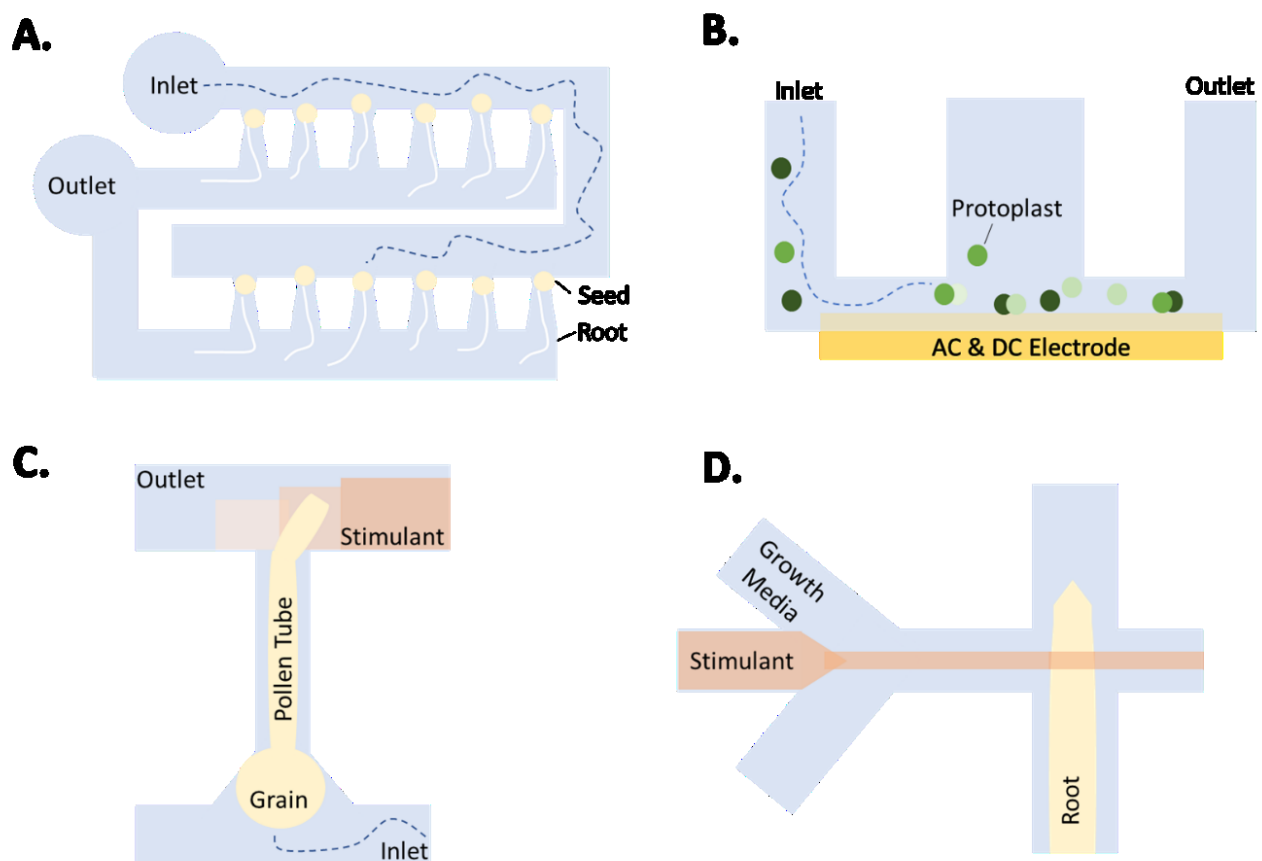


Figure 1.19 Microfluidics for Plant Cell Science. A variety of microfluidic technology has been implemented for investigating plant analysis. (A) Plant-chips, allowing seed placement through hydrodynamic trapping, followed by germination within the microfluidic device, allow real-time monitoring of root growth for phenotypic insights. (B) Electrofusion devices allow superior cell manipulation through AC pulses. Followed by precision in the application of gene transfection studies after the implementation of DC pulses for electrofusion. (C) Furthermore, microfluidics allows the precise creation of stimulant gradients, which provides directional insight of pollen tube growth (D) or precise local treatment of a stimulant to a growing root.

1.8 Thesis Outline

This thesis is composed of three parts with an overarching theme of furthering the contribution to knowledge in biological variability by implementing single-cell protein measurements. The first section is comprised of the miniaturised microfluidic assay methods followed by the results obtained in plant protein quantification experiments. The second section discusses novel applications in microfluidic technology and fluorescence microscopy towards increasing the throughput of the miniaturised microfluidic assay. The third section summarises this thesis's contributions to absolute protein copy number measurements in organelles, plants, and mammalian cells with an emphasis on expression variability and suggestions for future work in the area. A more detailed summary of each chapter is provided below.

Chapter 2 explains the materials, methods, and statistical applications for the MAC chip. Specifically, the development of the microfluidic design and optimisation of the miniaturised assays with regards to an increase in assay sensitivity. The approach in assay quantification through fluorescence microscopy and single-molecule localisation methods are explained. While the growth, isolation, and integration of single plant cells and organelles are detailed. The chapter concludes with a proof of concept experiment, pulling together these methods to address the alterations in chamber volume and the quantification abilities of this novel single plant cell protein quantification platform through comparing its quantification ability to that of epi-fluorescence microscopy.

Chapter 3 details and employs a novel tri-molecular assay in combination with the previously discussed microfluidic technology to measure the absolute copy number of Rubisco during the maturation of single plant cells isolated from *S. oleracea*. Further experimentation presents the ability to assess single organelle Rubisco copy numbers. Furthermore, this tri-molecular assay is expanded towards interrogating the protein-protein interaction of Rubisco with Rubisco Activase.

Chapter 4 extends this application of developing a tri-molecular assay within the microfluidic technology towards 4-HPPD copy numbers in single *A. thaliana* cells. While multiple antibody pairs are evaluated, the dynamic range of the tri-molecular assay failed to provide the requisite limit of detection for the copy number of 4-HPPD within a single cell. Highlighting fundamental limitations of antibody assay sensitivity in performing proteomic measurements in single cells. This chapter concludes with bulk cell measurements upon healthy and etiolating *A. thaliana*, providing an estimate for the single cell 4-HPPD copy number.

Chapter 5 begins the discussions of increasing the throughput of the miniaturised microfluidic assay. The original implementation of optical trapping to facilitate the isolation of single cells is tedious and time-consuming, so this chapter explains a method to overcome this. Through the fabrication of reduced volume microwell arrays with a previously developed tri-molecular assay for detecting p53 in mammalian cells to increase the throughput of protein copy number measurements. While theoretically, this platform would constitute a throughput increase we see that actual experimental limitations hinder this throughput.

Chapter 6 furthers the throughput of the MAC chip absolute copy number measurement technology by employing epi-fluorescence microscopy. This is achieved first by the quantification of three fluorescent cell lines through both MAC chip methodology and epi-fluorescence image acquisition. The concordance presented between these techniques facilitates the implementation of a fluorescent secondary standard, allowing absolute copy number quantification in epi-fluorescence microscopy.

Chapter 7 explores the gamma distributions created from cell-to-cell variance in protein copy number expression for the proteins measured within this thesis. We demonstrate that the absolute noise in protein expression, a total of both intrinsic and extrinsic noise sources, does not scale with natural protein abundance. However, upon a comparison of our total noise measurements with previously published quantitative proteomic data inclusive of total noise from both *E. coli* and *S. cerevisiae*, we find global scaling between the variance and mean in protein expression.

Chapter 8 summarises this thesis's contributions towards advancing the field of quantitative plant biology. Specifically, through the development of new technologies for single plant cell analysis and the contributing knowledge to this field by the implementation of these technologies for quantifying both single organelle and single-cell protein copy numbers. This chapter also presents suggestions for future work upon these technologies for continued advancements in this field

Section 1:

A Miniaturised Microfluidic Technology Allowing Quantification of Plant Protein Copy Numbers

Chapter 2: Basic Principles & Technique

Chapter 3: Single Cell to Organelle Rubisco Quantification

Chapter 4: Bulk Cell Analysis of 4-HPPD

Chapter 2

Basic Principles & Techniques

This chapter outlines the Microfluidic Affinity Capture Chip workflow, with an emphasis on the materials and methods permitting quantitative plant proteome investigation. The preliminary steps in selecting a microfluidic design is based upon a posteriori knowledge of fluid dynamics and the soft lithography methods to actualise this microfluidic design is highlighted. The development, detection, and statistical analyses of the integrated protein quantification assay are detailed. Followed by the materials and methods for plant growth, isolation, and integration within this microfluidic device. The chapter concludes by fitting together these techniques into a proof of concept experiment addressing the quantification abilities of this novel plant cell protein quantification platform in comparison with epifluorescence measurements.

2.1 Introduction

Cellular analyses enabled by microfluidic devices is a rapidly expanding field, this is in part due to the important metamorphosis of single-cell biology, originally a philosophical afterthought and now a major area of biomedical research [117]. This quantitative interest towards the variability in single-cells has created an explosion of new technologies, many based on microfluidic components, due to the advantages discussed in Chapter 1, relating to small volumes and the ability to integrate a variety of functional elements. One such technology motivated by this quantitative biomedical research explosion was a microfluidic device developed by Salehi-Reyhani et al. [78]. This device is capable of single cancer cell analysis and is composed of microfluidic analysis chambers which contain a capture antibody spot for single-cell measurements.

To respond to the gap in single plant cell research, as proposed in section 1.7.1 the approach mentioned above detailing antibody capture spots in microfluidic assay chambers has been modified and applied towards providing a different “single-cell” perspective for plant biology research. The fundamental microfluidic handling and measurement technology was adopted due to previous literature, presenting the volume advantage for single cell

analysis and the recognition of antibody spot measurement systems being a valued technique. Specifically, the antibody spot studies have provided evidence towards the importance of choice in fabrication technology such as contact, lithography and non-contact antibody array fabrication technology [118,119]. Contact printing technology has been further examined providing evidence that the choices of antibody print buffers and the concentration of antibodies printed per spot present a direct impact upon the antibody capture performance while the assay volumes that the antibody arrays operate within can alter the detection limits [120,121].

2.2 Microfluidic Design and Fabrication

This section discusses the preliminary design and the subsequent microfluidic device fabrication. A device pattern, as shown in *Figure 2.1 A*, is the crucial beginning to the actualisation of a microfluidic device. Preliminary modelling of the fluid-structure interactions through computer-aided engineering allows the careful selection of a suitable pattern before fabrication. Once an ideal pattern has been selected, it is turned into a master mould by implementing conventional photolithography methods, that is employing optical means of transferring a device pattern onto a silicon substrate, as shown in *Figure 2.1 B*. This process is also referred to as soft lithography since elastomeric rubber materials are used. These soft lithography fabrication methods were initially developed in 2002 by George M. Whiteside and his student J. Cooper McDonald [6]. The design and fabrication of the MAC Chip is no different to a majority of microfluidic devices, such that preliminary validation of the device design is undertaken, followed by the employment of soft lithography processes for microfluidic device fabrication.

2.2.1 Characterization of Fluid Flow in the MAC Chip

The fluid dynamics within a microfluidic device is descriptive of laminar flow as no turbulence occurs, due to the small channel dimensions. This non-turbulent fluid flow can be quantified by the Reynolds number (Re), a unit-less quantity, which relates the fluid density (ρ), average velocity (V_{avg}), and viscosity (μ_v) within the hydraulic diameter (D).

$$Re = \frac{L V_{avg} \rho}{\mu_v} \quad (2.1)$$

A Reynolds number $<2,300$ indicates a non-turbulent flow, and within this regime, the volumetric flow is analogous to an electronic current [122]. Allowing the movement of molecules to be characterised through the comparison of electrons within an electrical circuit, such that each microfluidic channel segment is an electrical resistor, and the fluidic resistance (R_{Fluidic}) of each microfluidic channel segment can be calculated by taking the quotient of the change in pressure (ΔP) with the volumetric flow rate (Q) as shown in Equation 2.2 [123].

$$R_{\text{Fluidic}} = \frac{\Delta P}{Q} \quad (2.2)$$

COMSOL Multiphysics is a simulation software which can be implemented to perform CFD simulations after importing a microfluidic device pattern (user-generated geometry) coupled with Equations 2.1 and 2.2, allowing the characterisation of the fluid-structure interactions. The MAC chip has been designed so that the main channel ($35,000 \times 1,000 \mu\text{m}$) would exhibit a lower fluidic resistance than the adjoining analysis channels ($4,000 \times 40 \mu\text{m}$) as shown in Figure 2.2 A. So that fluid flow within the main channel would not disturb the fluid within the analysis channels.

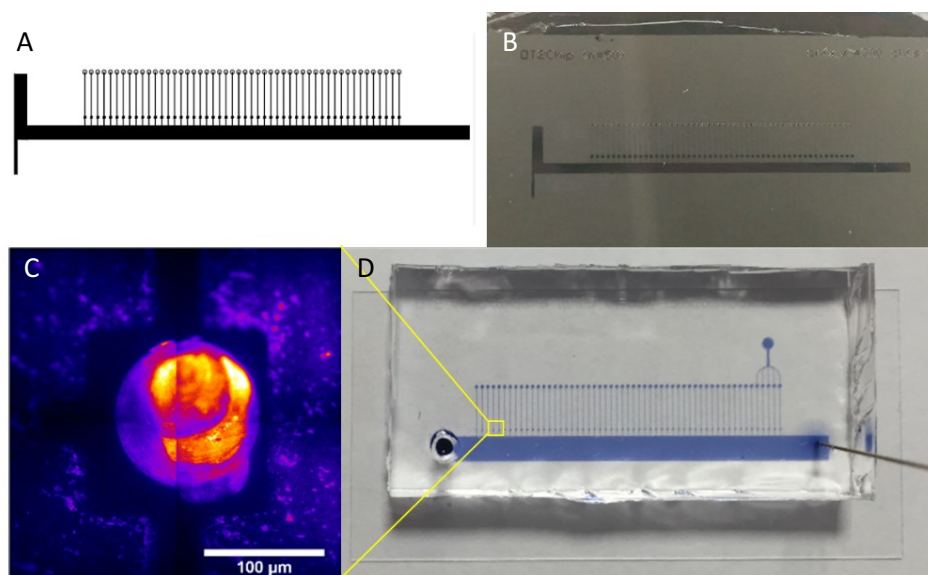


Figure 2.1 Microfluidic Device Fabrication. The MAC chip is fabricated using soft lithography techniques. (A) Where a mask containing the microfluidic features is placed upon a 4-in silicon wafer that has been coated with a polymerizable photoresist. (B) After exposure to ultraviolet light the unexposed pattern remains producing the mold. (D) PDMS is poured upon the mold and after curing for 2-days is removed producing a microfluidic device which is depicted by trypan blue vacuum infiltration allowing visualization of the microfluidic features. (C) Printed antibody spots were aligned into a 150×150 micron chamber, the printing buffer has been diluted with TRITC conjugated streptavidin to enable visualization by a tile scan image illuminated by TIRF.

This fluidic prediction has been simulated within COMSOL using a CFD simulation. This simulation was set to the values for the channel's hydraulic diameter, taken from the microfluidic device pattern. This pattern was filled with stationary PBS ($\rho = 1.06 \text{ g/cm}^3$ $\mu_V = 0.01 \text{ P}$) and its flow was simulated under the withdrawn average velocity ($V_{\text{avg}} = 1 \mu\text{L min}^{-1}$) from the outlet of the device pattern, representative of how a syringe pump would behave. We see in *Figure 2.2 B* that the velocity profiles within the main channel were higher than those generated within the analysis channels, conducive of the higher fluidic resistance promoted by the geometrical change within the analysis channels. These simulations provide theoretical evidence that if flow is induced within the main channel, it will result in little to no flow applied to the analysis channels.

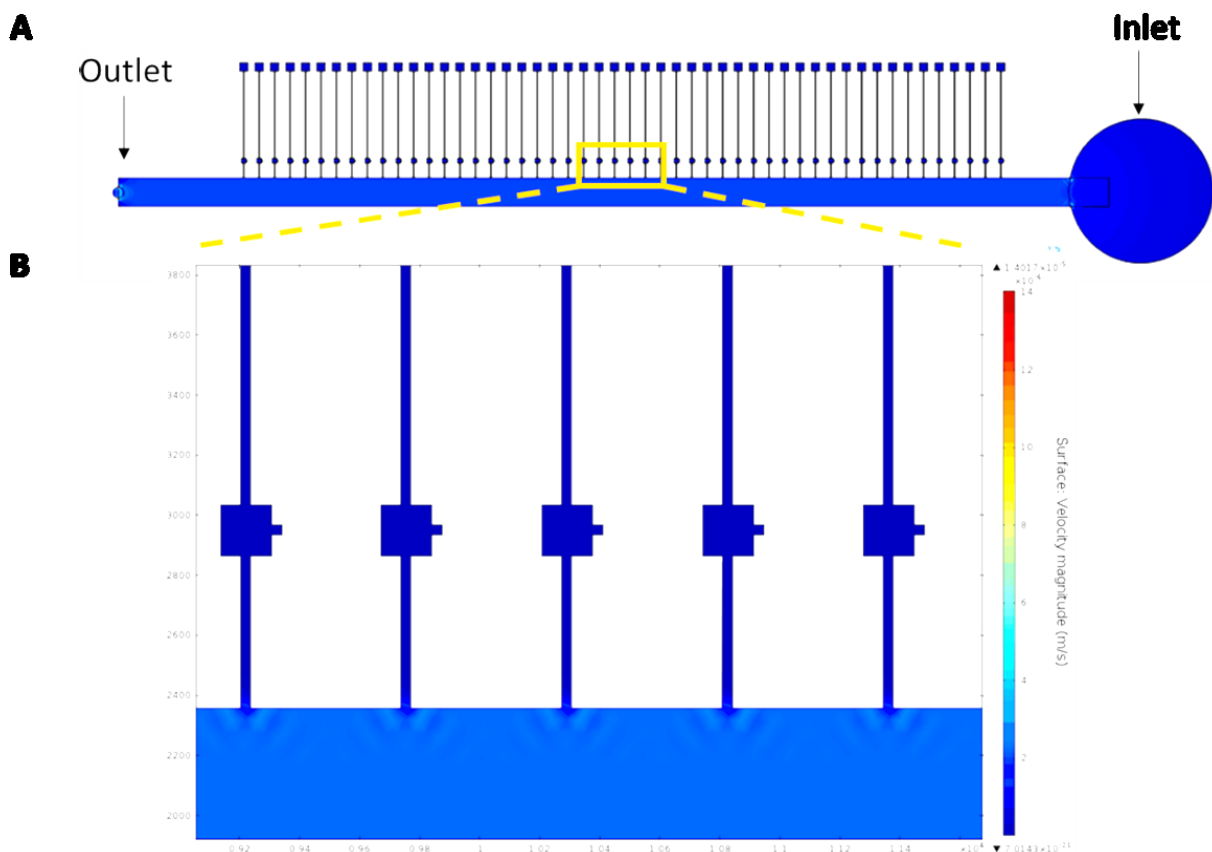


Figure 2.2 CFD Simulation in MAC Chip. (A) The geometry of the MAC chip including the 2.5mm inlet and 375 μm outlet was modeled in CAD software and imported with extremely fine mesh representing the fluid domains. Upon the withdrawal of fluid from the outlet at $1\mu\text{L min}^{-1}$ it is apparent the velocity of the fluid is faster in the main channel than that of the adjoining analysis channels. (B) This is displayed in a magnified view showing the fluid velocity boundary created between the lower fluid resistant main channel and the adjoining analysis channels.

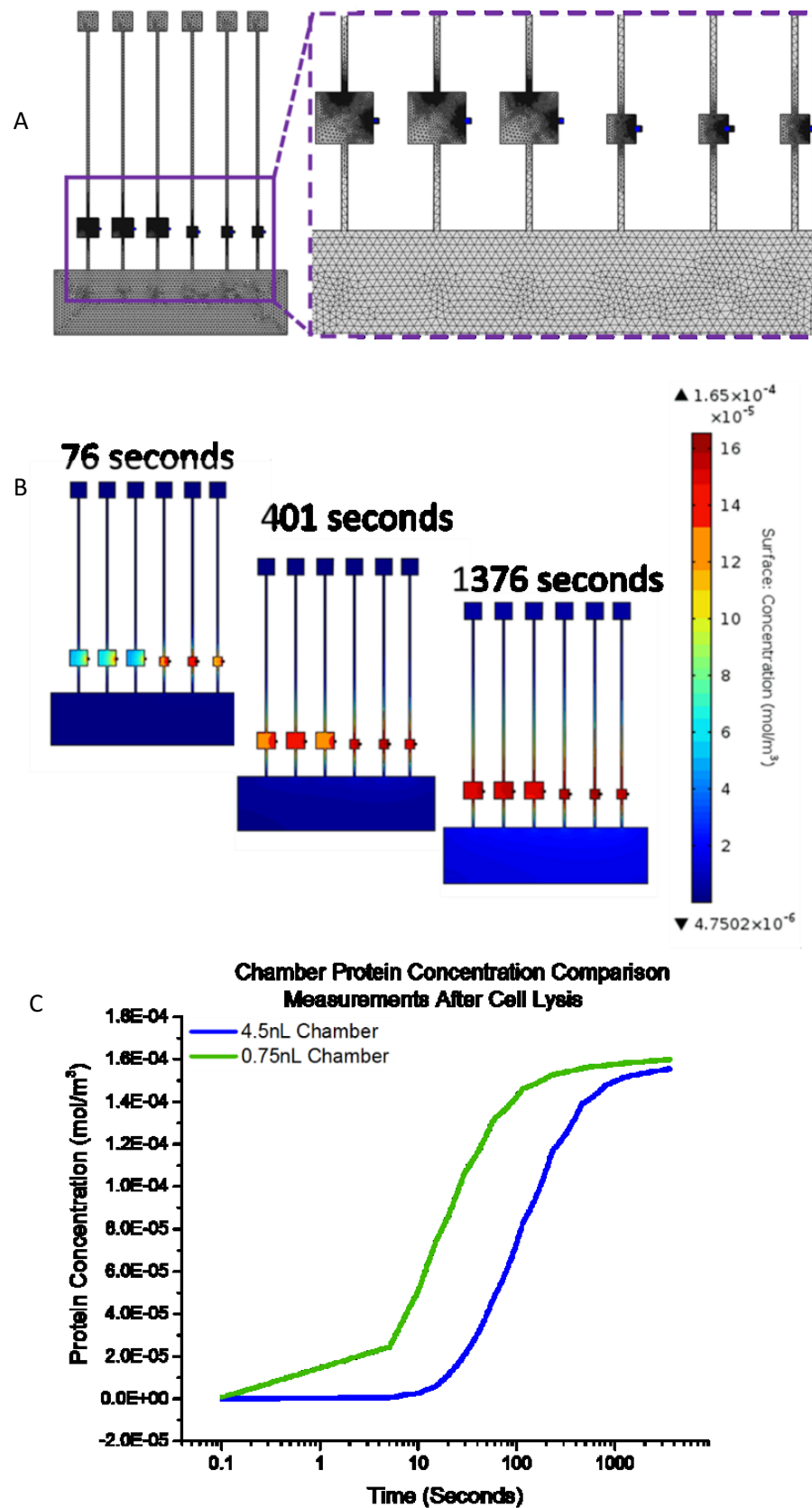


Figure 2.3 CFD Simulation of Diffusion in MAC Chip. (A) Upon rendering in fine geometry, the construction of three 0.75 nL and three 4.5 nL analysis chambers, each harboring a single cell. (B) The diffusion of protein concentration can be modeled from these single cells, similar to after cell lysis in the MAC chip. (C) It has been modeled and measured over the given timecourse that by decreasing the chamber volume the concentration equilibrium within the chamber is reached at a far quicker time course.

2.2.2 Characterization of Diffusion in the MAC Chip

Another important aspect towards the movement of molecules within a microfluidic device is diffusion because diffusion becomes the dominant force for movement of molecules within the MAC Chip after fluid flow from the syringe pump has ceased. Diffusion, calculated by *Equation 2.3* is the movement of molecules driven by a Brownian motion from an area of high concentration to a low concentration which can best be characterised by Fick's Law of Diffusion [124].

$$\frac{\partial \varphi}{\partial t} = D \frac{\partial^2 \varphi}{\partial x^2} \quad (2.3)$$

Fick's Law contains one parameter, the diffusion coefficient (D). When D is known for a molecule, the molecular rate of the diffusion process can be modelled in a dimensional concentration (φ) when the location (x) and time (t) have been specified. Diffusion of small molecules, such as GFP, within the MAC chip, can be modeled in COMSOL by implementing this partial differential equation coupled with the previously calculated diffusion times of GFP [125].

The simulation was run under the previously expressed values for PBS, being the solution within the microfluidic pattern. While the diffusion coefficient ($D = 87 \mu\text{m}^2 \text{s}^{-1}$), determined from the mobility of GFP in PBS is applied. Each cell before lysis contains $1.65 \times 10^{-4} \text{ M}$ of GFP, and the locational restrictions of GFP diffusion is rendered from the microfluidic pattern. Within these simulations, represented in *Figure 2.3*, we show that through decreasing chamber volume the concentration of the analyte (GFP) liberated from a single-cell can reach a maximum concentration at a ten-fold higher rate within the smaller chambers. Specifically, maximum concentration by diffusion in 0.75nL chambers can be obtained in 3 minutes, while in 4.5nL chambers maximum concentration is reached within 33 minutes.

2.2.3 Microfluidic Device Fabrication

After characterisation of the fluid dynamics within this pattern, the MAC chip is fabricated using established soft lithography procedures. Such that SU-8 2050 negative photoresist (Microchem, USA) was spun to a thickness of $\sim 35 \mu\text{m}$ on a 4-in silicon wafer using a spin coater (Laurell Technologies Corp., North Wales, PA). The coated silicon wafer was treated

with a pre-bake, placed on a hot plate at 65°C for a 2-minute soft bake followed by a 4-minute hard bake at 92°C, where the photoresist undergoes a reflow. A mask that contains the microfluidic patterns shown in *Figure 2.1 A*, was created using AutoCAD LT 2008 (Autodesk, San Rafael CA), placed on the wafer and is exposed to ultraviolet light (ThermoOriel, Stratford CT) polymerizing the exposed photoresist. Next, the wafer undergoes a post-bake process, placed on a hot plate at 65°C for 2-minutes followed by a 4-minute hard bake at 92°C. The unpolymerized photoresist is removed using SU-8 developer, 2-(-1-methoxy) propyl acetate (Sigma Aldrich, UK), rinsed with isopropyl alcohol and blown dry with nitrogen *Figure 2.1 B*. After wafer fabrication, a (10:1 w/w) mixture of polydimethylsiloxane (PDMS) prepolymer and curing agent is degassed under vacuum. Poured over the mould and cured for two days at room temperature, circumventing the shrinking of microfluidic features by heat induced curing.

The cured PDMS is removed from the silicon wafer, excess PDMS from the microfluidic features is trimmed off, and a biopsy punch is used to produce an inlet of 2.5 mm in diameter, outlets were drilled using a 1.4 mm drill bit. Following this, the PDMS is washed vigorously with a detergent solution of 1% Alconox to remove any debris. Then the microfluidic features, specifically the assay chambers, were aligned with antibody spots which have been micro-printed upon a coverslip *Figure 2.1 C*. A custom-built 6-axis alignment jig performs this alignment.

2.3 Assay Optimisation

The methods used for quantifying proteins are in essence a miniaturised ELISA assay contained within a ~nanoliter environment. Two assay approaches were used to quantify proteins within plants. A bimolecular assay, where the protein of interest was fused with a fluorophore or has fluorescent properties and is captured by the antibody spot, as illustrated in

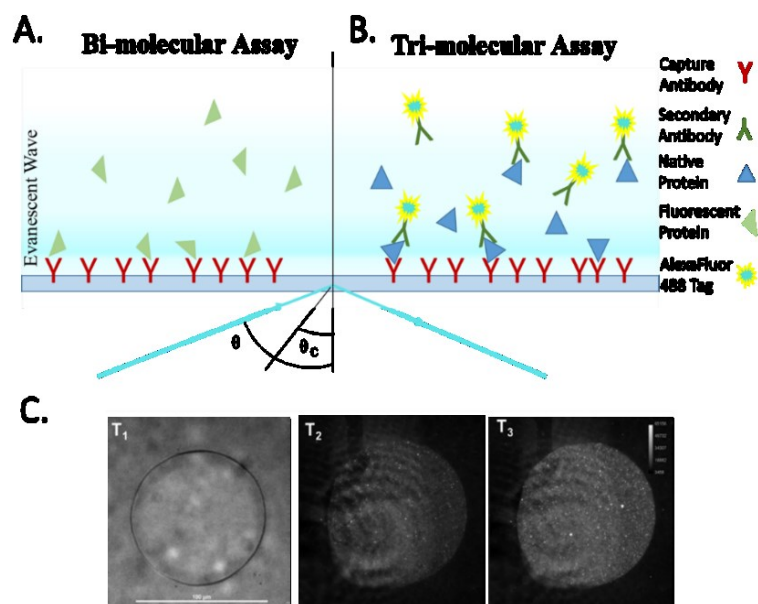


Figure 2.4 Protein Capture & Detection. (A) The Cartoon characterization of a bimolecular and (B) tri-molecular assay illuminated by TIRF for protein quantification. (C) Time course measurements of single molecule binding kinetics for a tri-molecular assay, only when equilibrium of the assay is reached do binding events reach maximum capture efficiency. Shows a brightfield illuminated antibody spot (C_{T1}) followed by the same antibody spot illuminated by TIRF imaged at 15 minutes (C_{T2}) and 60 minutes (C_{T3}).

Figure 2.4 A. The other approach is a tri-molecular assay, where a secondary antibody conjugated with a fluorophore is used for detection of the protein captured by the antibody spot, displayed in *Figure 2.4 B.*

Assay optimisation, the ability to detect the smallest quantity of protein, is obtained through altering all aspects of these assays, such as microfluidic chamber volume, printing surface of the coverslip, capture spot morphology, capture antibody conjugation, secondary antibody concentration or choice of the conjugated fluorophore. The purpose of the following will be to acquaint the reader with a broad set of conditions that can be applied to antibody assays, while further details of the conditions for specific protein assays are described in Chapter 3-section 3.3.3, Chapter 4-section 4.2.3, Chapter 5-section 5.2.3, Chapter 6-section 6.2.2.

2.3.1 Surface Passivation of Coverslip

To prevent fouling of the coverslip during an experiment, from nonspecific binding of biomolecules from cellular lysate and secondary detection antibodies a surface passivation method previously applied for coverslips has been adapted [126]. By implementing the following technique, an increase in the sensitivity of the assay can be achieved by decreasing the background noise produced by coverslip fouling that contributes to the overall statistical analysis. At room temperature (20° C) coverslips undergo cleaning through the immersion and sonication of 1M KOH, followed by milliQ water, then acetone, and finished in 1M KOH. Coverslips were prepared for mPEG-PEG Biotin functionalization by passivation with APTES, through immersion in a solution of (1mL APTES/ 5mL Acetic Acid/ 100mL MeOH) for 30 minutes, followed by rinsing coverslips with MeOH and milliQ water. Following this, coverslips were sandwiched together with a solution of (1mM sodium bicarbonate/ 5-15mg PEG-Biotin/ 50mg mPEG) and left in a humid environment for 3 hours and rinsed with milliQ water. After mPEG-PEG Biotin functionalization, a final layer of NeutrAvidin is applied (0.1mg/mL NeutrAvidin in PBS) using the same coverslip sandwich technique and left in a humid environment for 1 hour and rinsed with milliQ water. Fully functionalized coverslips were printed upon immediately.

2.3.2 Antibody Binding Kinetics

The ability to achieve single-cell quantitative proteomic measurements is reliant upon the affinity of the chosen antibodies implemented within the assay. This antibody affinity can be quantified by the equilibrium dissociation constant (K_D) a measurement of the interaction

between the antibody and its antigen. This interaction of a protein (P) and an antibody (A) undergoes dynamic binding events of collisions (k_{on}) and dissociation (k_{off}).



When these reactions are at equilibrium, that is the concentrations do not change; the mass action law can calculate the reactions

$$\frac{d[AP]}{dt} = [A] \cdot [P] \cdot k_{on} - [AP] \cdot k_{off} = 0 \quad (2.5)$$

Allowing the thermodynamic equilibrium to be defined as

$$[A] \cdot [P] \cdot k_{on} = [AP] \cdot k_{off} \quad (2.6)$$

This equation can be rearranged to define the equilibrium dissociation constant (K_D) as

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[A] \cdot [P]}{[AP]} \quad (2.7)$$

This equation can be rearranged to present evidence that the smaller the K_D value, the higher the affinity of complex formation $[AP]$ of the antibody $[A]$ for its antigen or protein $[P]$.

$$[AP] = \frac{[A] \cdot [P]}{K_D} \quad (2.8)$$

As the affinity is a constant (K_D) then the measurement of the fraction of protein bound (F_{bound}) can be a direct readout of either the amount of protein (P) in the system when the number of (A) is constant or the number of antibodies (A) in the system when the number of (P) is constant. This relationship can be shown by

$$F_{bound} = \frac{[AP]}{[A + P]} \quad (2.9)$$

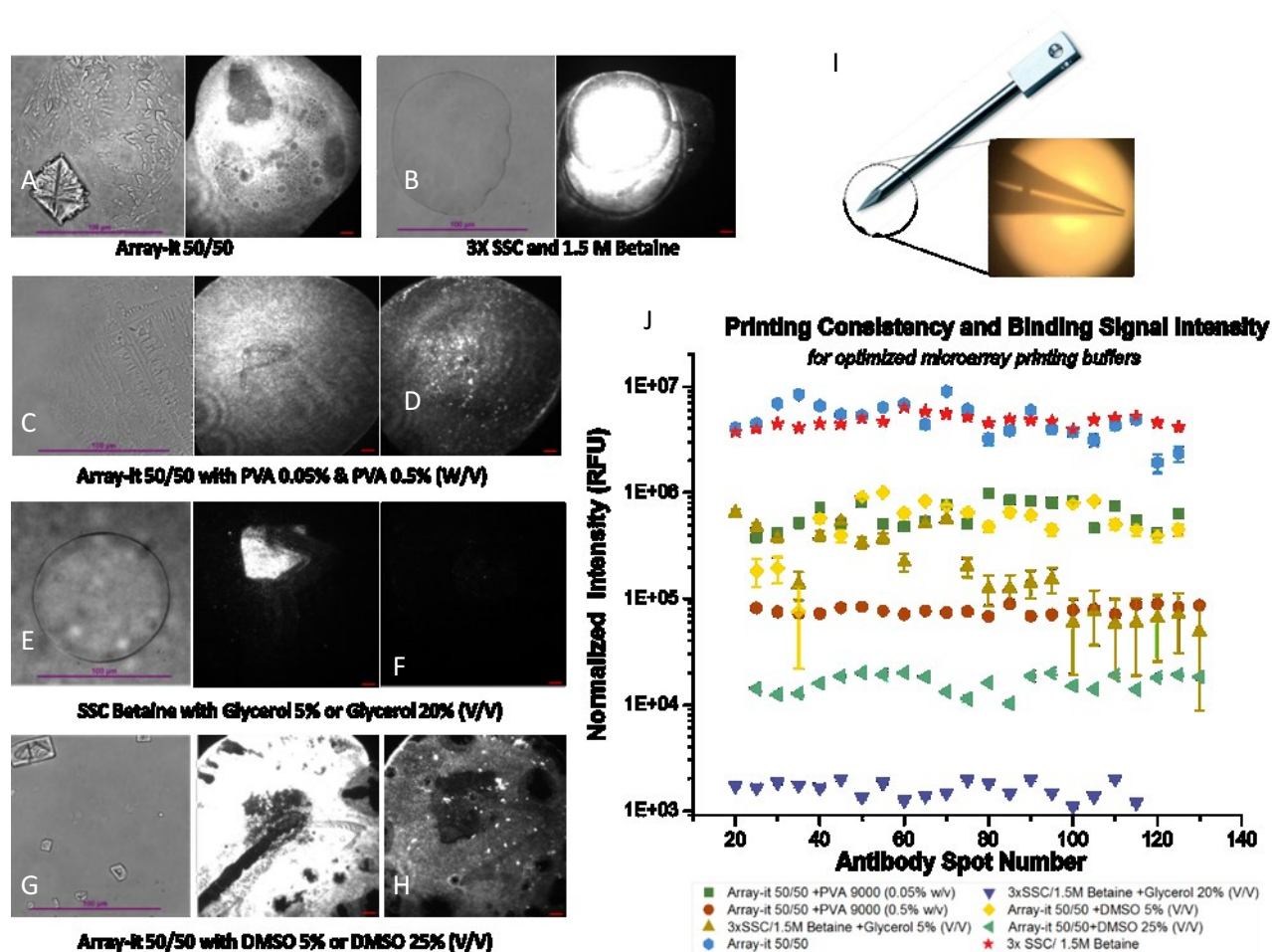


Figure 2.5 Print Additives and Antibody Microarray Reproducibility. Antibody spot morphology and reproducibility is subject to the microarray printing buffer, the concentration of antibody in the printing buffer and the printing environment and technology used. For the above microarrays, the same mouse antibody was printed at 0.5mg mL^{-1} and only alterations in the microarray printing buffer was changed such that Array-it (A) 3xSSC and 1.5M betaine (B) Array-it diluted with PVA 0.05% (C) or PVA 0.5% (D). 3X SSC and 1.5M betaine diluted with Glycerol 5% (E) or Glycerol 20% (F). Also, Array-it diluted with DMSO 5% (G) or DMSO 25% (H). The pin printing technology used was 946MP2 pins purchased from Array-it (I). The pin was filled one time with antibody diluted with printing buffer and printed over 130 spots. (J) A graph depicting the reproducibility of antibody immobilization from each printing run, the intensity corresponds to the binding of secondary anti-mouse antibody conjugated with FITC.

2.3.3 Capture Antibody Spot

Microarrays were fabricated using a quill-type pin printing technology [118]. Specifically, a 946MP2 pin (Array it, USA) depicted in *Figure 2.5 I*. The first step towards optimization of antibody spot printing performance is through the choice of print buffer (Array-it or 3X SSC 1.5M Betaine) and the additional print additives (Glycerol, PVA, 0.1% SDS) that can be mixed into the original antibody solution. Print buffers and print additives change the spot deposition by modifying solution viscosity, surface tension, and evaporation rates [127]. The performance of print buffers and print additives have been shown to be antibody dependent, that is, the printing buffer for antibody A may perform differently for antibody B.

To determine spot morphology and spot-to-spot heterogeneity preliminary experiments were performed by printing an antibody that was raised in a mouse (Enzo Life Sciences, UK) and utilising all available printing buffers shown in *Figure 2.5*. These spots were covered with a 150 μ L solution containing 1×10^7 molecules/nL of anti-mouse FITC conjugated secondary antibody in 4% BSA in PBS. After the interaction between the antibodies reached equilibrium images were obtained and analysed for capture spot fluorescence intensity, analysis is shown in *Figure 2.5 J*. As can be seen through this experiment, spot-to-spot and intra-spot heterogeneity can be varied concerning additives and print buffers, which would not be ideal for protein quantification. The print buffer 3X SSC 1.5M Betaine and Array-it outperformed with respect to signal intensity. Therefore, a higher density of antibody immobilised, and a greater spot-to-spot signal homogeneity was obtained. This is ideal for protein quantification purposes. Furthermore, the addition of PVA produces high intra-spot homogeneity, an overall larger spot size, yet decreased the overall signal intensity. Unadulterated Array-it was favoured over Array-it with PVA due to the latter problems.

Furthermore, altering printing concentrations of the capture antibody from 0.5 mg/mL to 1 mg/mL directly affects the amount of antigen captured, as seen in *Figure 2.6 A*. Such that increasing the concentration of printed antibody increases the number of available antibodies for antigen capture until steric hindrance is achieved within the capture antibody spot causing a reduction in antigen capture *Figure 2.6 D*. However, due to the proclivities of antibody distributors, it has been very uncommon to find antibodies sold at such high concentrations, greater than 2mg/mL, which would exhibit the steric hindrance described here.

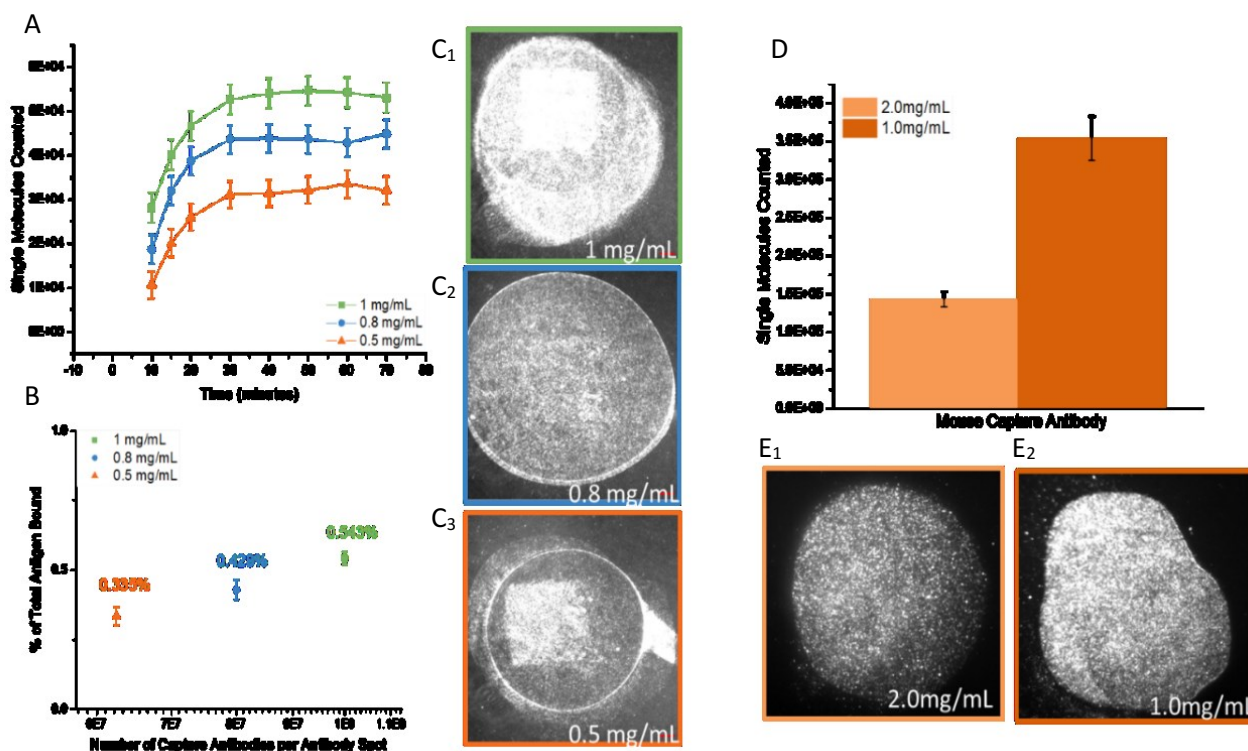


Figure 2.6 Alteration in Capture Antibody Concentration. Equilibrium of an antibody assay is determined by the antibodies dissociation constant K_D . (A) Manipulating the concentration of antibody in the capture spot does not change the time necessary to reach equilibrium. (B) However, it does have direct implication upon the percentage of antigen bound. Experimental images are shown for a capture antibody at equilibrium for 1 mg mL^{-1} (C₁) 0.8 mg mL^{-1} (C₂) and 0.5 mg mL^{-1} (C₃). (D) Steric hindrance, whereby antibodies prevent neighboring antibodies from binding an antigen, can be achieved with high antibody printing concentrations as seen with a mouse capture antibody. Experimental images from a steric hindered antibody spot printed at 2 mg mL^{-1} (E₁) and optimal printing at 1 mg mL^{-1} (E₂).

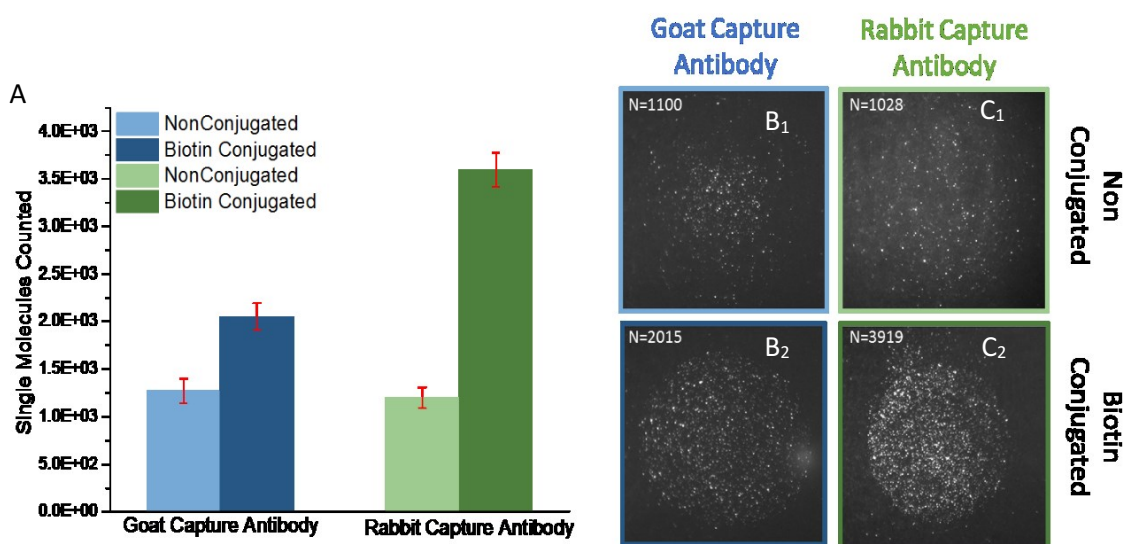


Figure 2.7 Conjugation of Capture Antibody. Producing favorably orientated capture antibodies by the method of conjugating Biotin with the F_C region of an antibody produces a greater amount of actively binding antibodies within a spot. (A) Comparing the before and after biotin conjugation we present a 4X higher capture efficiency for a rabbit antibody while only a 1.75X increase with a goat antibody. This can possibly be explained by the amino acid sequence variability within the F_{AB} region of the goat antibody producing less favorably orientated capture antibodies. Experimental images of the goat capture antibody before (B₁) and after Biotin conjugation (B₂). Experimental images of rabbit capture antibody before (C₁) and after Biotin conjugation (C₂).

2.3.4 Secondary Detection Antibody

When creating a tri-molecular assay, a secondary detection antibody is implemented. The secondary antibody affinity for antigens can be altered by choice of the conjugated fluorophore, we have seen evidence of this not only within our own MAC chip calibration studies which have been characterized in section 5.4.2, with the binding curve data presented in *Figure 5.13*, but also through information obtained by BioCore upon the same purchased secondary fluorophore-conjugated antibodies presented in *Figure 5.12*. Furthermore, the concentration of secondary antibody within the chamber alters the limit of detection, thereby enhancing or decreasing the signal to noise within the assay. For all work performed throughout this thesis, unless specified, the preferred concentration is 1×10^7 molecules of secondary antibody per nL.

2.3.5 Antibody Validation

Western blots were performed to validate the specificity of the antibodies implemented in the assays. The first step is performing cell lysate creation. This was achieved through the addition of 1X RIPA buffer (Abcam, UK) at 0.5 mL per 2.5 million cells. Quantification of cells/mL was performed through hemocytometer counting (Fisher Life Science, UK). After the addition of RIPA buffer, cells were placed on ice for 15 minutes, then centrifuged at 14,000 rpm for 15 minutes. Supernatant was removed, and stored on ice as solution C, while the pellet was resuspended in 0.5 mL 1X RIPA buffer and resolubilized through sonication and labeled as solution M. Solution C contains the cytosolic proteins and solution M contains the membrane proteins, equal parts are mixed and stored on ice until being loaded on the SDS-PAGE gel.

Proteins were separated on SDS-PAGE gels (10% SDS gel NuPAGE; Thermofisher, UK). Known cell concentrations (cell/mL) per sample were mixed (3:1) with 4x Laemmli sample buffer (Bio-Rad, UK). After mixing the samples were heated in a hot water bath at 70°C for 10 minutes, before being loaded into the wells on the SDS-PAGE gel. SDS-Page was performed in a MOPS SDS Running Buffer (Invitrogen, UK) at a constant voltage of 150 V for 1 hour with a start current of 40-55 mA/gel and an end current of 25-40 mA/gel.

After proteins have been separated and organised based on protein weight, the cassette which encloses the SDS-PAGE gel is removed with the utmost of caution. The western blot portion of this procedure began by equilibrating the gel in blotting buffer (50 mL NuPage Transfer Buffer, 100 mL Methanol, 850 mL deionised water) for 15 minutes within a polyethene container (Rubbermaid, UK). The sponge and pads were also presoaked within

this container in blotting buffer. The transfer setup is built within this container, and all parts were submerged in blotting buffer. The setup was produced as – ground, sponge, blotting paper, gel, nitrocellulose membrane, blotting paper, sponge, positive current- take care to not move or smear the gel with the nitrocellulose membrane as proteins begin to transfer immediately. This setup was transferred into the western blot chamber, which is filled with blotting buffer, and the current was set for 20V for 2 hours and 30 minutes.

The nitrocellulose membrane was removed and incubated in blocking solution for 30 minutes. Blocking solution was composed of 10x TBS (24.23g Tris, 80.06g NaCl, mixed within 800mL of deionised water and brought to a pH of 7.6 with HCl), this was mixed as (100mL of 10x TBS, 900 mL of deionized water, with the addition of 1mL tween-20) then 5g of BSA per 100 mL of TBST solution created the final blocking solution. Nitrocellulose was removed from the blocking solution and rinsed in TBST 3x by incubation of 15mL of TBST for 5 minutes each rinse.

To probe the nitrocellulose gel for protein identification, the nitrocellulose was immersed in a solution of the chosen fluorescence antibody for 2 hours, or overnight if necessary. Before the addition of the antibody, it is diluted in TBST at the manufacturer's suggested dilution or 1:500. Following this, the nitrocellulose was rinsed in 15mL of TBST 3x for 5 minutes each before the nitrocellulose was imaged for in gel-fluorescence.

2.4 Assay Detection

Single molecule detection through optical methods, inclusive of Total Internal Reflection Fluorescence (TIRF) Microscopy was first demonstrated by Tomas Hirschfeld in 1976 [70,128]. This work was significant for it showed the possibility of detecting labelled proteins. Specifically, γ -globulin conjugated to a fluorescent polymer, without the need for enzymatic, chemical measurements. Single molecule analysis is advantageous as the results obtained are not inherent of ensemble averaging. Therefore, one can adequately characterise the population and even detect small or rare differences within a fluorophore population [129].

The methods employed within the MAC Chip for fluorophore illumination upon the capture antibody spot consist of using TIRF microscopy coupled with a perfect focus system (PFS) integrated within a Nikon TI-E microscope (Nikon, Japan) allowing the focal plane of illumination upon the antibody spot to be maintained. After image acquisition, a digital readout of binding events is obtained using single-molecule localisation microscopy previously utilised in super-resolution microscopy from the University of Sussex [130].

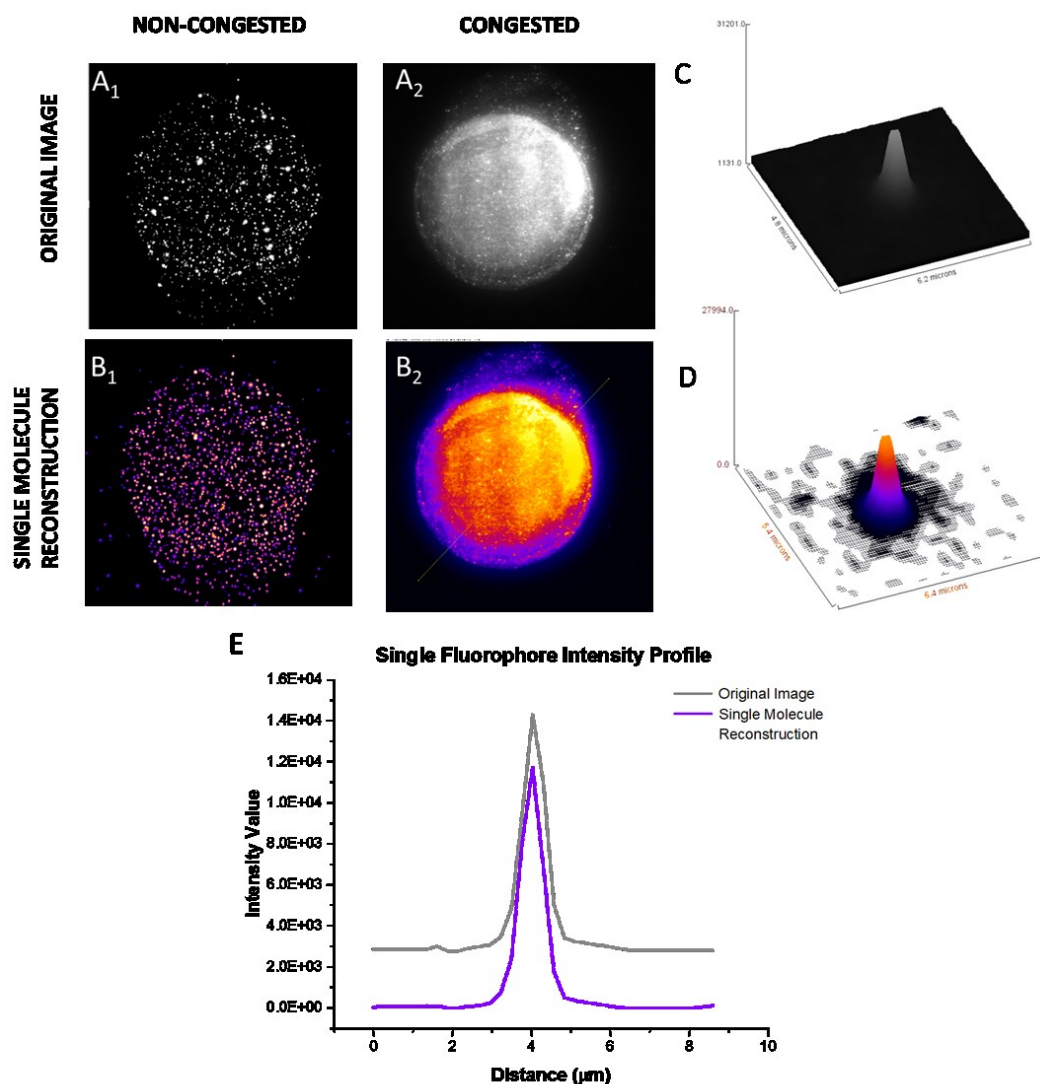


Figure 2.6 Single Molecule Identification and Quantification. The ability to quantify single fluorophores bound (A_1 A_2) in an original antibody assay image to produce a (B_1 B_2) digital read out of the total number of single molecules present is dependent upon a super resolution microscopy software adapted from the University of Sussex. (C) Original imaged fluorophores undergo laser profile background subtraction (D) producing unadulterated approximated single 2D Gaussian peaks which are quantified per image, a comparison of peak intensity is presented (E). Digital quantification is applied to non-congested spot images (A_1) while an analog method, by which, the image is divided by the mean intensity of a single fluorophore is applied to congested spot images (A_2).

2.4.1 Experimental Platform

A fully motorised inverted Nikon Ti-E microscope (Nikon, Japan) was used as the central platform in all experiments using a 60x (NA=1.49) oil immersion objective (Nikon, Japan). The location of molecules or cells for image analysis can be encoded into the XY stage (Nikon, Japan) while the PFS automatically corrects focus drift in real time during extended periods of image analysis. This microscope is capable of both widefield microscopy and TIRF microscopy allowing respectively, both widefield white-light or fluorescence imaging and single molecule imaging.

The excitation light sources implemented for TIRF microscopy were either a 20 mW 473 nm continuous wave laser (MBL-473-200 Laser 2000, UK) or a four colour Stradus VersaLase system (Vortran Laser Technology, USA) allowing excitation output at 405 nm, 488 nm, 532 nm, and 647 nm. Each laser system was fibre coupled through a single mode fibre to the motorised Nikon TIRF illumination system (Nikon, Japan) allowing the correct angular orientation for TIRF microscopy. Widefield light sources were provided by halogen and mercury lamps (Nikon, Japan). Fluorescence detection and all images were recorded by an IXON, DU-897E electron multiplying charged-coupled device (EM-CCD) (Andor Technologies, Ireland).

2.4.2 Total Internal Reflection Fluorescence Microscopy

To illuminate fluorophores that were bound to the capture antibody spot TIRF microscopy is utilised. This technique has been widely applied for single-molecule imaging [130,131]. The process of total internal reflection is generated from incident light waves refracting and reflecting at the coverslip interface (oil-coverslip and coverslip-media), as shown in *Figure 2.4 A*. This originates from the waves propagating into a medium with different indices (n_1 and n_2), specifically a medium of oil into media. At and above a critical angle (θ_c), which can be calculated from Snell's law in *Equation 2.10*, the reflection of the light waves will be greater than 90°.

$$\theta_c = \sin^{-1} \left(\frac{n_2}{n_1} \right) \quad (2.10)$$

While a majority of the incident light is refracted back into the originating medium (n_1), a small amount penetrates the boundary surface creating an evanescent field of excitation light that extends 100-200 nm from the coverslip surface into the second medium (n_2). Thereby almost exclusively exciting the fluorophores located upon the coverslip surface, while reducing the background from the fluorophores in solution. The penetration depth of this evanescent wave decays exponentially with respect to the z-axis. When the light intensity at the interface (I_0) is known, then the light intensity ($I(z)$) at a certain penetration depth (d), with a perpendicular distance (z) from the interface of the two mediums can be calculated from *Equation 2.11*.

$$I(z) = I_0 e^{-z/d} \quad (2.11)$$

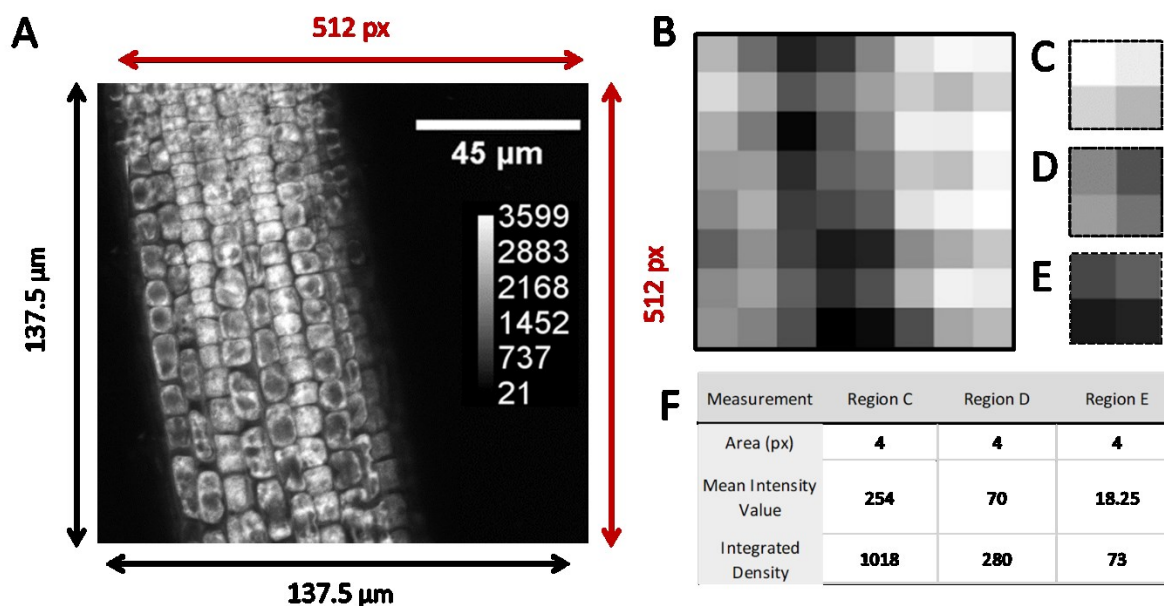


Figure 2.7 Spatial and Intensity Image Measurements. (A) Fluorescence microscopy acquired image of *A. thaliana* basal meristem cells expressing GFP within a root. This image can be interrogated for size measurements as the number of pixels and their distance is known, permitting a scale bar (45 μm). The intensity of fluorophores in the image is represented by the LUT bar (21-3599), where the shade of grey intensity is associated with a pixel numeric value. (B) When zooming into a small section of this image it is composed of small squares of different shades of grey. Each square corresponds to a pixel, which encodes a numerical value which corresponds to the LUT bar. For fluorophore intensity measurements, such as in the four-pixel regions (C, D, and E) selected from B. (F) The regions can be measured for a mean intensity value for all four pixels, this corresponds to the concentration of fluorophore. Another measurement is the integrated density, where all pixel intensities are summated, this corresponds to the absolute concentration of fluorophore in the measurement area.

2.4.3 Single Molecule Counting

To quantify how many fluorophore binding events have taken place within the capture antibody spot at equilibrium, a program previously implemented at the University of Sussex, for single molecule localisation microscopy has been adapted by Dr Alastair Magness to suit the needs for non-congested and congested spot analysis *Figure 2.8 A₁ and A₂* respectively. In the non-congested analysis, single fluorophores shown in *Figure 2.8 C* are witnessed as diffraction-limited intensity peaks and approximated to an ideal 2D Gaussian function. However, when a substantial portion of these intensity peaks become unresolvable due to overlap, the images undergo congested analysis, where the image is divided by the mean intensity of a single fluorophore derived from control chambers on a per experiment basis.

2.4.4 Spatial and Intensity Measurements in Image Analysis

The smallest unit which composes an image is a pixel; each pixel corresponds to a number, which in turn can be displayed as a coloured square corresponding to the measurement of light intensity spatially defined within an image, displayed in *Figure 2.9*. Our captured images, using a 60x objective, are displayed by 262,144 pixels in a rectangular array of 512 x 512. The

total image distance is 137.5 μm . Therefore, the pixel width and height are both $137.5/512 = 0.27 \mu\text{m}$. Through knowing this real-world pixel value images can be calibrated (0.27 μm /pixel) allowing size measurements of a specimen within the image. Measurements of size are performed through scientific image analysis software, Image J.

The intensity (coloured square) displayed by each pixel is determined from lookup tables (LUTs), a table which defines the pixel values alongside the possible colour used to display them. Adjusting the LUT causes the pixel values to remain unchanged, yet the colour associated with the value is modified. The intensity of a group of pixels within an image, specifically within a specimen can be measured, through Image J, to produce the mean intensity value and integrated density value. Respectively, these values correspond to the concentration of light intensity and the total amount of light intensity within the group of pixels measured. In this thesis, the light intensity corresponds to a fluorescent molecule. Therefore, these measurements correspond to the concentration of fluorescent molecules or the total amount of fluorescent molecules within the area of the captured image.

2.5 Methods in Statistical Analysis

Statistical methods allow the extraction of information from raw research data through the implementation of mathematical formulas, models, and techniques. This thesis employs OriginLab for the production of scientific graphs and statistical analysis of the research data in exploring the absolute quantification of proteins, the variance within populations of cells, and assessing the agreement between quantitative methodology.

2.5.1 Population vs Sample

A population is composed of observations that could continually be taken from a range of objects (i.e. all leaves in St. James Park). As populations can be composed of an infinite group of objects only a subset of this population is usually measured for an observation (i.e. 500 leaves from St. James's Park). This subset is the definition of a statistical sample, and when objects are chosen without bias, this can be representative of the population. This bias is critical to account for, as creating a flawed statistical sample can occur through accidental sampling (i.e. only leaves which are at eye level are chosen) causing a statistical sample not to be genuinely representative of the population.

Observations, in the context of biology, can be classified into two types: measurement variables and nominal variables. Measurement variables are individual observations which can be numerically classified, such as leaf weight, length, and pH. A nominal variable is a

word, not a number, such as the species of leaves collected in St. James Park. Observations can also involve a single variable (univariate data) or two variables (bivariate data) and may respectively be assessed in the variability of the observation or assessing relationships between two observations.

2.5.2 Univariate Data Analysis

Central tendency or the *measures of location* are the values that compose a sample described by the mean ($\bar{\mu}$) and median (m), that is the average value and middle value. The mean is calculated by the summation of all values (x_i) in the sample divided by the number of values in the sample (n) and is an estimate for the population mean (μ).

$$\bar{\mu} = \frac{1}{n} \sum_{i=1}^n x_i = \frac{x_1 + x_2 + \dots + x_n}{n} \quad (2.12)$$

Dispersion of observations or *measures of variation* around this sample mean are formed from the basis of the sum of squares (SS) calculated by subtracting the mean ($\bar{\mu}$) from an observation value (x_i) and squaring this deviate, followed by summing these squared deviates.

$$SS = \sum (x_i - \bar{\mu})^2 \quad (2.13)$$

When the sum of squares is divided by the number of values in the sample (n-1), the sample variance (v^2) is calculated, providing a measure of the spread of values with respect to the average [132]. This sample variance is an estimate for the population variance (σ^2), as it is rare that measurements can be taken for the entire population.

$$v^2 = \frac{\sum (x_i - \bar{\mu})^2}{(n - 1)} \quad (2.14)$$

To provide a measure of this dispersion in its original units, not squared units, the square root of the variance is taken providing the sample standard deviation.

$$\sigma = \sqrt{\frac{\sum (x_i - \bar{\mu})^2}{(n - 1)}} \quad (2.15)$$

This standard deviation then allows us to estimate how far the sample mean ($\bar{\mu}$) is likely to be from the population mean (μ). This estimate is achieved by taking the standard deviation (σ) and dividing the squared number of values in the sample (n) calculating the standard error (σ_x^-) of the sample mean.

$$\sigma_x^- = \frac{\sigma}{\sqrt{n}} \quad (2.16)$$

Another measure of dispersion in sample observations around the average is the coefficient of variation (η), defined as the sample standard deviation (σ) divided by the sample mean ($\bar{\mu}$). This summarizes the amount of variation as a percentage of the total, independent of units. Making this measurement very useful when comparing the amount of variation among samples with different mean values.

$$\eta = \frac{\sqrt{\frac{\sum (x_i - \bar{\mu})^2}{(n-1)}}}{\bar{\mu}} \quad (2.17)$$

Probability distributions are mathematical functions that forecast the probabilities of finding values (x_i) which would exist within a population based on sample measures of location ($\bar{\mu}$) and variation (v^2). The shape of a distributions can be described by moments about the mean and presented as

$$\frac{1}{n} \sum_{i=1}^n (x_i - \bar{\mu})^r \quad (2.18)$$

The exponent r is described as the r^{th} moment. The first moment is equal to the mean. The second moment about the mean is variance. The third moment is related to the skewness and the fourth moment is related to the kurtosis of the distribution [133]. The skewness is descriptive of the tailing in the distribution, such that a distribution presenting tailing to the right would be identified as skewed to the right, while the kurtosis of a distribution is a measurement of the relative flatness in a distribution under comparison with what is presented by a normal distribution.

Probability distributions are generally divided into two classes, either discrete probability distribution or continuous probability distributions. The focus is on the latter, as the histograms produced in this thesis describe the occurrence of continuous values. The likelihood of finding a value (x) in a continuous distribution that represents the measured variable (X) between two points is predicted through a probability density function (PDF) defined as $P(X)$ and summarized as

$$P(X = x) = p[a \leq x \leq b] = \int_a^b f(x)dx = 1 \quad (2.19)$$

The integral of the probability density function is 1, which arises from the property of discrete distributions in which the sum of all the probabilities must equal one.

There are many probability distributions ($P(x)$) which all exhibit different r^{th} moments as described in *Equation 2.18*. Some examples are the Weibull Distribution, Exponential Distribution, Lognormal Distribution, and Normal Distribution. When chosen appropriately these PDF's can be viewed as tools for dealing with uncertainty, such that the probability (p-value) of finding a measurement for a variable can be defined. Simple distributional measurements can obtain confidence in the choice of fitting these probability distributions to the measured variable (X). Such as a visual comparison of the actual data (F_n) with that to a fitted distribution (F), through the implementation of quantile-quantile plots. A slightly more sophisticated test is to compute the momentums of F_n (mean, standard deviation, skewness, and kurtosis) and evaluate a hypothesis towards known momentums in (F) to characterise the data.

The Anderson-Darling test is one such test, which is used throughout this thesis to validate if F_n is a normal distribution, log-normal distribution, Weibull distribution or exponential distribution. Through assessing the test statistic (A) for a given distribution against that of the given critical values assigned for the univariate data distribution. The test statistic is defined as

$$A^2 = -N - S \quad (2.20A)$$

In which N is the sample size and S can be solved for by the cumulative distribution function (F) of the specified distribution and the standardised values in the univariate data distribution (F_i). Standardized values are produced by

$$F_i = \frac{x_i - \bar{\mu}}{\sigma} \quad (2.20B)$$

S can be solved by

$$S = \sum_{i=1}^N \frac{(2i-1)}{N} [\ln(F(F_i)) + \ln(1 - F(F_{N+1-i}))] \quad (2.20C)$$

This test was chosen for its ability to measure the distance between F and F_n with a higher weight on observations in the tails of the distribution in comparison to the Kolmogorov-Smirnov test.

2.5.3 Bivariate Data Analysis

The purpose of bivariate data analysis is to assess relationships between two variables (X, Y) which is usually encompassed by one nominal variable (i.e. leaf size (X) and leaf vein area (Y) for a nominal variable of plant species in St. James Park). These relationships can be described by their *association*, meaning that if measured X in the ith individual changes does the Y variable for the same individual tend to change. A measure of this joint variability is covariance and plays an essential role in not only bivariate but also multivariate statistics. This measure is defined as the expected product of the variable deviations from expected values given as

$$cov(X, Y) = \frac{1}{N} \sum_{i=1}^N (x_i - \bar{\mu}_x) (y_i - \bar{\mu}_y) \quad (2.21)$$

Where sample mean, and standard deviation are denoted by the x or y subscript, and i denotes an individual in the data set. This variance can be normalised to scores between -1 and 1 to allow comparison between the amount of association among samples with different values. This total association of the data when normalised is defined as the Pearson correlation coefficient (*r*)

$$r = \frac{1}{N-1} \sum_{i=1}^N \left(\frac{(x_i - \bar{\mu}_x)}{\sigma_x} \right) \left(\frac{(y_i - \bar{\mu}_y)}{\sigma_y} \right) \quad (2.22)$$

A value of +1 identifies a perfect positive linear correlation, 0 is no linear correlation, and -1 is a perfect inverse linear correlation. Linear regression can represent these correlated relationships between X and Y. Where a line of best fit, calculated from the data values, is set closest to all the data points to provide the best general representation of the data set obtained from X and Y. The general form of this linear regression for bivariate data is

$$y = a + b_{yx}x \quad (2.23)$$

In which X and Y represent the variables, (a) is the y-intercept, and (b_{yx}) is the regression coefficient. The regression coefficient represents the rate of change in y as a function of a change in x. It is important to note that a correlation between two variables (X, Y) does not imply causation, a measurement of causation can be assessed between the strength of the relationship between these variables.

Describing the *strength* of the relationship between the bivariate data is achieved by examining the points on the graph and their distance to the actual regression line, this distance or fraction of unexplained variability is called a residual. Residuals can be calculated by subtracting the model estimates from the actual data and can be summarised by the coefficient of determination (R^2). This value provides information for the goodness of fit and ranges from 0 to 1, where 1 indicates the regression correctly predicts the real data points. Calculated, as shown in *Equation 2.22*, by dividing the residual sum of square (SS_{res}) by the total sum of squares (SS_{tot}), the latter being proportional to the total variance of the data as calculated by *Equation 2.13*.

$$R^2 = 1 - \frac{SS_{res}}{SS_{tot}} \quad (2.24A)$$

$$R^2_{adj} = 1 - \frac{s^2_{res}}{s^2_{tot}} \quad (2.24B)$$

The coefficient of determination can be adjusted (R^2_{adj}) as shown in *Equation 2.23 B*, by assessing the variance of the sample rather than the sum of squares. This method is useful when comparing the suitability between regression models, as it adjusts the number of explanatory terms in a model relative to the number of data points.

2.5.4 Comparative Data Analysis

Data sets composed of either univariate or bivariate data can be compared through a variety of methods. Before comparison, the importance of including the units of measurement should be identified, such that if different measurement techniques produce different units of measure, then the preservation of units is unnecessary and comparing these data sets should be performed independently from the units of measure. The ability to decouple the units of measurement has previously been presented when calculating the coefficient of variation (η) for a data set as shown in *Equation 2.17*. This normalisation of data is also seen when each measurement (x_i) in a data set (X) is divided by the mean value of the data set such that

$$x_i(X) = \frac{x_i(X)}{\mu(X)} \quad (2.25)$$

This method is employed quite frequently in this thesis and is identified as *normalised to mean*. This is employed primarily when comparing methodology which produces altered units of measure or when variations around the mean are multiplicative, such that data is best fit to be viewed on a logarithmic than a linear scale. Employing this method allows the level of variation around the mean to remain unchanged.

Another approach to transforming data for sample comparison is the creation of z-scores for each value (x) in a data set (X). This process of standardisation is composed of two techniques. The first involves the subtraction of the mean, which preserves the units of measurement for comparison, this has been identified through the thesis as *standardised mean subtraction* and alters each value as

$$x_i(X) = x_i(X) - \mu(X) \quad (2.26)$$

The ability to acquire a z-score (z_i) is achieved by taking *Equation 2.25* and dividing this by the standard deviation of the sample. When this method is applied on data within this thesis, it is identified as *standardised*. This transformation produces comparable distributions through scaling by the standard deviation, allowing independence from units. The mean value is 0 while the standard deviation is 1 for all standardised samples.

$$z_i = \frac{x_i(X) - \mu(X)}{\sigma(X)} \quad (2.27)$$

One of the most commonly implemented methods for data set comparison is the analysis of variance (ANOVA). Fundamentally, this test is applied for assessing the equality of the means through investigating the collective variance. Through asking does the variance of the combined data sets equal the variances of the individual data sets, allowing one to gain insight towards the significance of the difference between data sets. A limit to this test, ANOVA, is that it may only be implemented for normally distributed data that obeys the rules of homogeneity in variance. Therefore, if homogeneity in variance cannot be assumed the Welch's ANOVA can be implemented or if the data is not normally distributed the Kruskal Wallis H Test can be applied. This test assesses the data sets similar to an ANOVA yet in place of the mean value it assesses the medians of two data sets for differences.

Another method for data comparison is comparing the variations in data sets, this is identified as a statistical F test (F) and is calculated as a ratio of the sample data set variances. Implying that population variances are equivalent if the F value is equal to 1. If units of measure need to be decoupled, a ratio of the coefficient of variation (η) can be employed to assess the percentage of variance between two data sets.

$$F = \frac{\eta(X)}{\eta(Y)} \quad (2.28)$$

2.6 Isolation of Single Plant Cells and Organelles

There are a variety of methods for single cell extraction such as protoplasting, mechanical isolation, laser microdissection and mechanical micromanipulation. Similarly, there is a wide diversity of single cell types found in the leaf, stem, flowers, and root that can be examined. We have opted for protoplasting, specifically a form of enzymatic cell wall digestion, due to the well-documented techniques and the reported understanding of dedifferentiation and photosynthetic competence in single cells after isolation. Furthermore, we chose to perform the enzymatic digestion of mesophyll cells from the leaf and columella cells from the root, due to the protein distributions we opted to investigate.

The ability to analyse plant cells in microfluidic devices can be achieved by germinating seeds within the channels of a microfluidic device allowing root analysis or monitoring cotyledon development [112,113]. Alternatively, to culture or transfect plant

cells within a microfluidic device, off-chip isolation of single plant cells is performed, and the resulting single cells are loaded into the chip (microfluidic device) where either the dedifferentiation and division of cells can be monitored or the fusion of protoplasts can be performed, respectively [115,116]. For the application of single cell analysis within the MAC chip, the latter method, such that single plant cells were isolated and introduced into the microfluidic device was chosen.

2.6.1 Plant Materials

Spinacia seeds (*Spinacia oleracea* Missouri F1; Homebase, UK), Arabidopsis seeds (Col-0 wild-type; Turnbull Lab, Imperial College London), and transgenic Arabidopsis seeds (Wave line 131 and 35S: GFP; European Arabidopsis Stock Centre) were incubated in darkness at 4°C for 2 days before planting. Seeds were sown into pots filled with a moist substrate composed of a 3:1 ratio of John Innes No 2 compost (Homebase, UK) and vermiculite (Homebase, UK). Seeds after germination and growth were sustained under long day conditions within a plant growth chamber, with 14 hours of light (350-400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), at 22°C and 10 hours of dark.

To generate root protoplasts, the plant growth was performed under sterile conditions to avoid compost contamination. Transgenic Arabidopsis seeds (TCSn:10; European Arabidopsis Stock Centre) were sterilised using 20% (v/v) bleach and 0.1% Tween for 5 min followed by rinsing 5x with sterile water. Sown on a Petri dish containing standard Murashige and Skoog medium (4.4 g/L, 1% sucrose, 0.5 g/L MES, 1% agar and adjusted to pH 5.7 with KOH). Seeds were incubated in darkness at 4°C for 2 days before being transferred into the aforementioned long-day conditions where they were grown vertically for 14-25 days.

2.6.2 Mesophyll Protoplast Isolation

Adapting from previously published techniques a protocol was derived for extracting protoplasts [17, 18]. Protoplasts are single plant cells composed of protoplasm and plasma membrane. This was achieved through the addition of cellulase and pectinase which degrades the cell wall through modification of cellulose and polysaccharides respectively. It has been shown that protoplasts isolated from Spinach leaves can maintain photosynthetic stability for up to 9 hours [136].

Leaves from these plants were sliced with a fresh razor blade to create 0.5 mm segments, which were dipped into an enzyme isolation media (400 mM Mannitol / 20 mM KCL / 10 mM CaCl / 0.3 g Macroenzyme R-10 / 0.08 g Cellulase Onozuka R-10 / bring to final

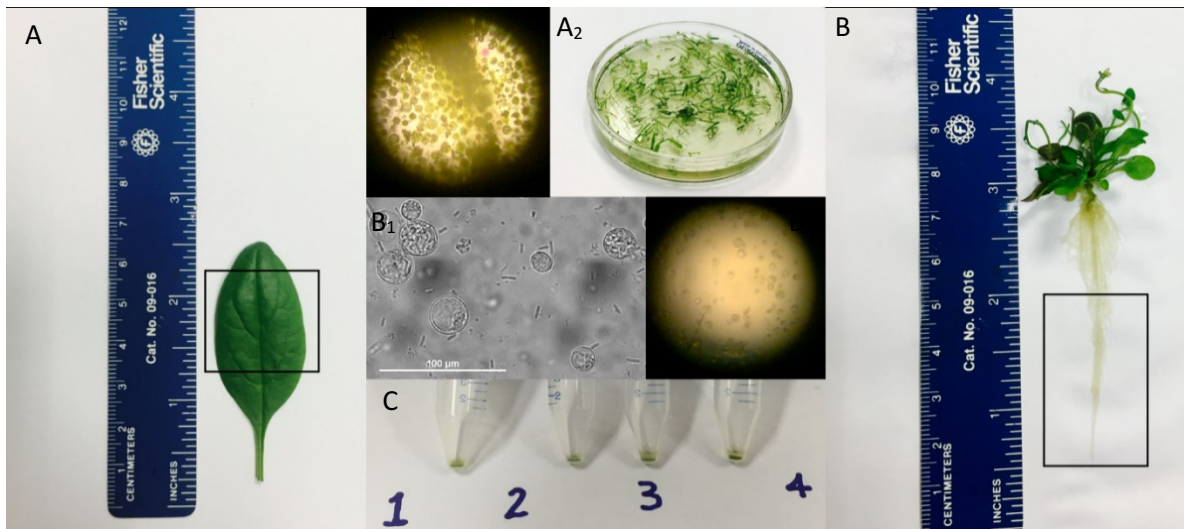


Figure 2.8 Isolation of Single Plant Cells. Single protoplasts were isolated by enzyme digestion methods, this is first accomplished by slicing with a fresh razor blade sections of 0.5 mm in the demarcated black boxed region for (A) leaves and (B) roots. (A₂) These sections are submerged in enzyme digestion media. After digestion of tissue segments and filtration the cells are purified by centrifugation methods, (C) the end result for leaves is a green pellet or for root tissue a white pellet. (A₁). 10X Brightfield image of protoplasts in enzyme digestion media. (B₁) 60X Brightfield image of isolated root protoplasts and (B₂) 10X Brightfield image of root protoplasts in enzyme digestion media.

volume of 20mL with Milli Q water). The media is then vacuum infiltrated into the leaf segments for 30 minutes and left to digest in a dark room for 3 hours, *Figure 2.10 A₂*.

After dark room incubation, the media is placed on an orbital shaker, at 75 rpm for 10 minutes to release the protoplasts. Media is then filtered using a 45 μm filter mesh and decanted into four separate centrifuge tubes. 1mL of W5 solution (4.5g NaCl / 9.2 g CaCl₂/ 185mg KCl / 495 mg glucose / 50 mg MES / brought to a final volume of 500 mL with Milli Q water and adjust to pH 6.0 with 0.5M KOH) is gently layered on top and tubes were centrifuged at 70g for 7 minutes. It is essential to note protoplasts are extremely sensitive towards high centrifuge speeds and rapid braking of the centrifuge rotor. Therefore, all isolation experiments were performed with the centrifuge brake disengaged. Following this a green line of isolated protoplasts were formed between the boundary of W5 and the enzyme isolation media, this is removed, washed in W5, centrifuged again at 50g for 5 minutes and resuspended in 4% BSA W5 and kept at 4°C until the initiation of MAC chip experimentation.

2.6.3 Root Protoplast Isolation

Root protoplast isolation was achieved analogous to the solutions and plant growth conditions in our previously implemented protocol and adapted concerning previously published methodology [91]. One-third of the root shown in *Figure 2.10 B* from Arabidopsis was dissected into 2 to 4 mm pieces and submerged into a flask containing the enzyme isolation media. The media was vacuum infiltrated into the root segments for 30 minutes. Following this, the root segments were incubated in the media for 2 hours in darkness upon

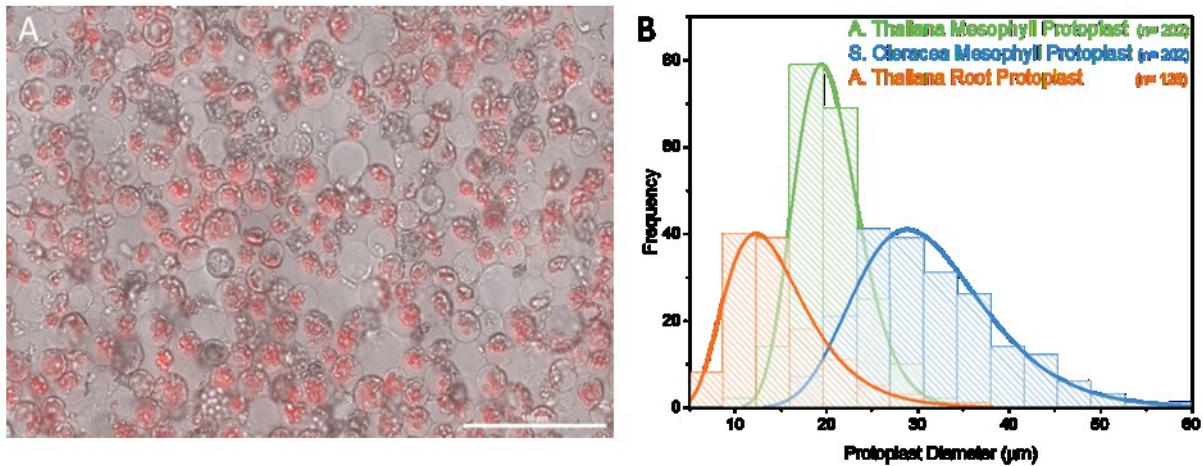


Figure 2.9 Protoplast Cell Size Comparison. To determine cell size a solution of isolated protoplasts is placed upon a coverslip then following sedimentation images were captured as shown in (A) scale bar is 100 µm. Where Arabidopsis protoplasts have been imaged in both Brightfield and in CY5 wavelength, owing to the red Autofluorescence witnessed from chloroplasts present within the protoplasts. The cell diameter can be measured by using the spatial measurement methodology discussed in section 2.4.4. It is shown that the average diameter for Arabidopsis protoplasts is 20.19µm while Spinach protoplasts were larger with an average diameter of 31.43µm. The average diameter of Arabidopsis root protoplasts is 14.74µm. (B) The distributions fit best to a lognormal distribution, as validated through an A-D test for Goodness of Fit.

an orbital shaker at 125 rpm. Protoplasts were separated from the undigested root tissue by using a 45 µm filter mesh, then centrifuged at 1000 g for 3 minutes. The resulting root protoplast pellet was resuspended in 4% BSA W5 and kept at 4°C until the initiation of MAC chip experimentation. There is a noticeable cell diameter variation for Spinach and Arabidopsis mesophyll protoplasts, which is displayed in *Figure 2.11 B*. The diameter measurements for all protoplasts, obtained by spatial measurements described in section 2.4.4, were best fit to a lognormal distribution for both populations.

2.6.4 Chloroplast Isolation and Double Membrane Validation

Chloroplast isolation was adapted from the previously described methods, where 30 g of spinach leaves grown in the conditions described in section 2.5.1 were homogenized in a blender within a solution of chloroplast isolation buffer (CIB) (0.33M Sorbitol, 20mM HEPES, 2mM EDTA, 1mM MgCl₂ and 0.1% BSA with a pH of 6.5) [137]. The macerate was filtered through 100 µm then 45 µm filter mesh. The resulting solution was centrifuged at 200 g for 3 minutes, and the supernatant was removed. The supernatant was further centrifuged at 1,000 g for 7 minutes providing a green pellet of chloroplasts. In addition to these methods of chloroplast isolation, a 40% Percoll gradient was applied to provide separation of ruptured and intact double membrane chloroplasts, providing only intact chloroplasts for MAC chip experimentation [138]. This was achieved by re-suspending the green pellet of chloroplasts in CIB and layering this suspension upon 20 mL of 40% Percoll. Centrifugation at 1,500 g for 7 minutes, and removing the supernatant which contains broken chloroplasts, then re-suspending the pellet in CIB with 4% BSA.

Chloroplast intactness was quantified by epi-fluorescent measurements validating the presence of a double membrane which is essential for isolating import-competent and photosynthetically active chloroplasts [139]. Double membrane validation was achieved by the addition of CFDA (0.005% w/v), a polar, cell-permeable, fluorescent dye which exhibits a strong fluorescence when de-esterified to carboxyfluorescein (CF). When cleaved the polar fluorophore remains within the stroma due to the intactness of the membrane. The chloroplast contains up to three carboxylesterases within the stroma which may be responsible for this de-esterification of the CFDA. Our results indicated that over 88% of chloroplasts isolated contained a double membrane. Images from chloroplast intactness experiments can be seen in *Figure 2.12*.

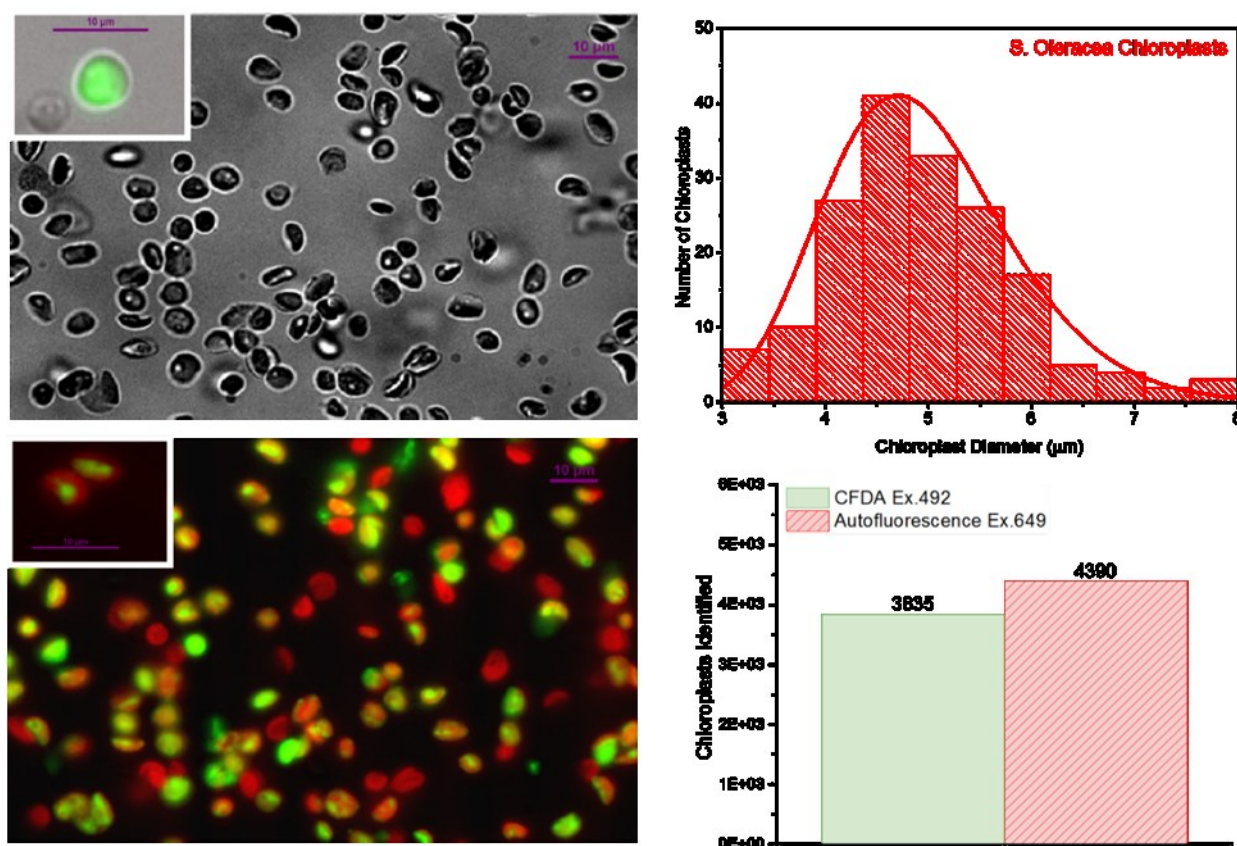


Figure 2.10 Chloroplast Double Membrane Validation. (A) Spinach chloroplasts viewed with phase-contrast microscopy and (B) fluorescent microscopy with fluorescein specific filter after incubation with CFDA. Intact chloroplasts produce a more pronounced halo feature, which corresponds with CFDA retention (inset A), furthermore CFDA uptake appeared highly localized in some chloroplasts (inset B). (C) Diameter distribution measurements from phase-contrast images of chloroplast size fits to a lognormal distribution. Implementing similar spatial parameters from phase-contrast images upon 649 excitation autofluorescence and CFDA fluorescence provided validation that 88% of chloroplasts isolated retained double membrane features (D) scale bar = 10 μm .

2.7 Integration of Plant Cells

2.7.1 Optical Trapping

In general, the ability to manipulate the isolated single plant cells or single organelles in a microfluidic environment can be achieved through various means [19]. The fabrication of microfluidic valves could be utilized to precisely control fluid flow within a microfluidic device thereby halting flow and allowing cells to be accurately trapped within an analysis chamber [108]. Another method would be the introduction of microfluidic structures that would allow the hydrodynamic trapping of single cells in precise locations while cells were transported under the laminar fluid flow [27,28]. However, these methods are conducive to manipulating fluid flow facilitating the manipulation of the single cells. To circumvent this and only induce movement upon a single-cell, optical tweezers were implemented and have been applied previously as a method to manipulate chloroplasts and peroxisomes within plant cells [140,141]. To manipulate single cells, ytterbium fibre laser (YLM-5; IPG Photonics,

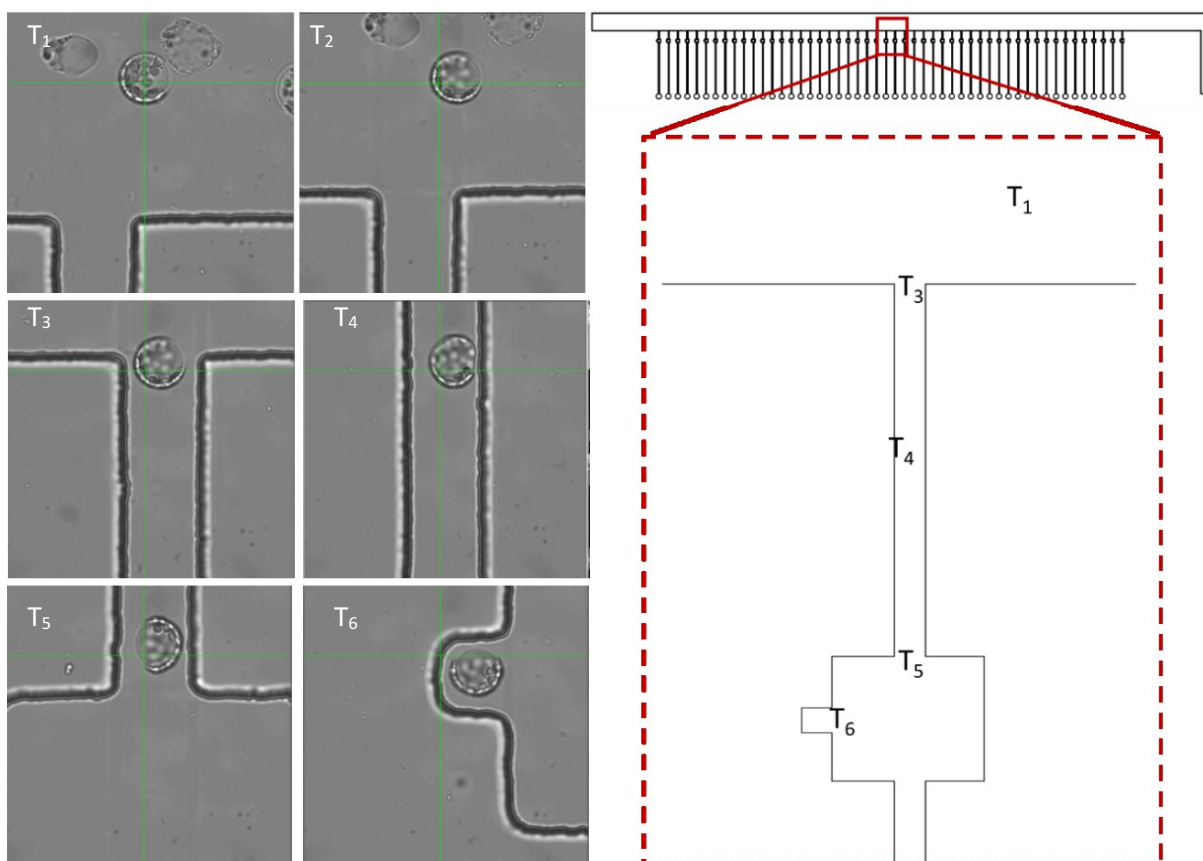


Figure 2.11 Video Frames from Optically Trapping a Protoplasts Video frames have been extracted (A) showing the first moments in cell reorientation as a chloroplast becomes focused within the laser (T₁-T₂). The microscope stage is translated in X and Y orientation allowing the trapped cell to be moved from the main channel into an analysis channel (T₃-T₅). Upon entrance into the analysis chamber the cell is placed within the holding cubicle (T₆) The green cross in all images denotes the location X and Y location of the laser beam. (B) A locational map of time points from the extracted video frames.

UK) with a power output of 0.5-1.0 W is introduced through the 60x objective and generates a single beam with the focal and trapping planes being coincident. After a cell is trapped, the cell is effectively moved by translating a motorised microscope stage. The technique of optical trapping works on the basis that the refractive index of the single cells is slightly higher than the surrounding media, thereby producing scattering forces that trap the single cells into the path of the optical force [142]. Using this methodology, we have shown the ability to trap and move single protoplasts and single chloroplasts in our microfluidic environment as shown in *Figure 2.13*.

2.7.2 Optical Lysis

Lysis techniques within microfluidic devices range from optical, acoustic, mechanical, electrical or chemical means. The selection of the most appropriate lysis technique is dependent upon the results one aims to achieve, such as for protein detection should the protein structure be native or denatured. For high temporal resolution, an ultrafast lysis technique should be employed [18]. Due to the constraints within our microfluidic device, we have chosen an optical lysis technique, to disrupt the protoplast membrane.

This first step, in liberating the proteins from a single protoplast is performed by the delivery of a single laser pulse of 6 ns at $\lambda=1064$ nm from a Q-sw Nd: YAG laser (Surelite SL I-10, Continuum, USA). This pulse produces a cavitation bubble which upon expansion exerts shear stress upon the protoplasts and causes dissociation of the cell membrane. Liberating the cytosolic proteins of interest in combination with the chloroplasts, and nucleus, as presented in *Figure 2.12*. This method has previously been shown to preserve cellular proteins and has been described in further detail elsewhere [143,144].

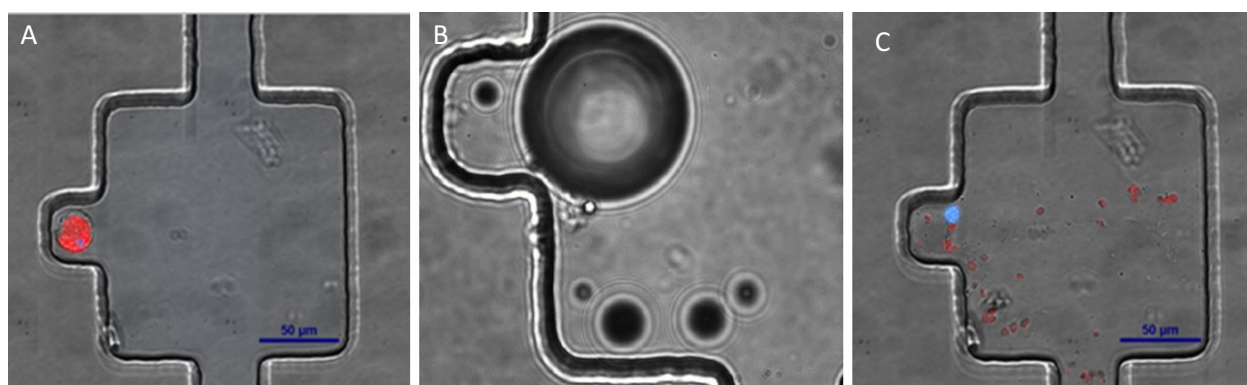


Figure 2.12 Images from Optical Lysis of a Protoplast. (A) After cells have been loaded into the cubicle within the analysis chamber (B) a single laser pulse of 6ns produces a cavitation bubble. (C) This promotes shear stress upon the cell membrane causing its rupture and the dispersion of chloroplasts and nucleolus respectively depicted as red spheres from chlorophyll Autofluorescence and a blue sphere from intercalation of DAPI. Scale bar= 50 μ m

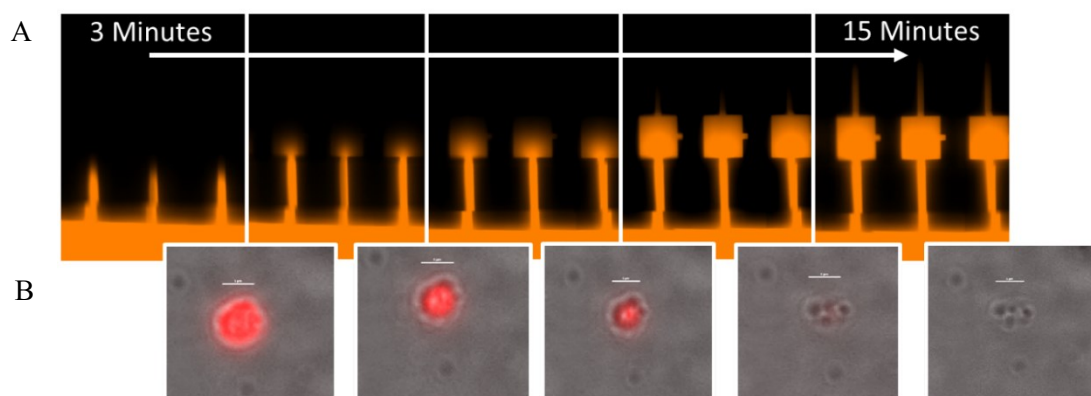


Figure 2.13. Chloroplast Chemical Lysis. 4X RIPA lysis buffer is loaded into the main channel, by means of diffusion the lysis buffer enters the analysis chamber and reaches optimal working concentration before 15 minutes. (A) The diffusion of Calcein into analysis chambers has been depicted to demonstrate the diffusion of small molecules within the MAC Chip. (B) Images of chloroplast lysis respective of the above diffusion time scale. Scale bar= 5μm

2.7.3 Integration of Lysis Buffer

To allow further analysis of proteins that are not within the cytosol, such as those within the chloroplast stroma, further lysis is necessary. Radioimmunoprecipitation assay (RIPA) buffer (Abcam, UK) at a concentration of 4X is flowed into the main inlet which then diffuses into the assay chambers facilitating the lysis of chloroplasts. From brightfield and epi-fluorescence images of the chloroplast membrane integrity over time we see that in 15 minutes after lysis buffer diffusion the chloroplasts undergo lysis and the stroma proteins from the chloroplasts were introduced into the assay chamber for capture and analysis, images of this can be seen in *Figure 2.15*.

2.7.4 Number of Chloroplasts per Protoplast

Through the implementation of optical lysis and the subsequent release of chloroplasts within the assay chamber, we show the ability to quantify how many chloroplasts are within a protoplast. Quantification of this chloroplast number is achieved by acquiring a focused tile-scan image of the assay chamber after sedimentation of chloroplasts upon the coverslip. These images were imported into Image J, and chloroplast numbers were quantified through the technique of thresholding. Thresholding removes the pixels in the image which are below the fluorescence intensity exhibited by a chloroplast, as shown in *Figure 2.16 A*. While the analysed particle feature in Image J counts the number of chloroplasts based upon the remaining pixel values which exhibit spherical and area attributes similar to that of a chloroplast. These images were also re-examined through visual quantification of the number of chloroplasts present in an assay chamber. We have seen in *Figure 2.14 D*, as previously shown, that chloroplast number scales with the development and expansion of a plant cell [145,146,147].

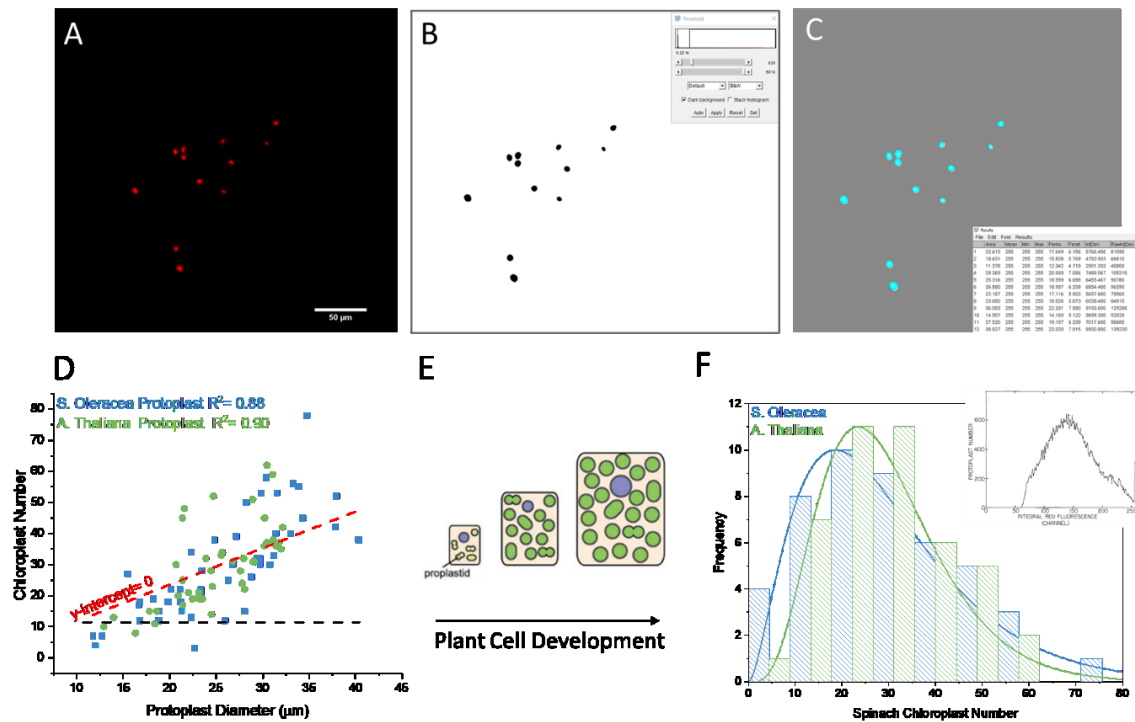


Figure 2.14 Chloroplast Number per Protoplast Diameter. A high correlation between plant cell diameter and the number of chloroplasts within the cell for Arabidopsis and Spinach has been measured, $R^2=0.90$ and 0.88 respectively. This was achieved by single cell isolation, protoplast membrane lysis, and (A) identification of the liberated spatially dispersed chloroplasts. (B) The technique of thresholding is applied to identify chloroplasts based on their chlorophyll autofluorescence generated by laser excitation at a wavelength of 649 nm. (C) The analyze particle feature in Image J recognizes chloroplasts based upon their spherical nature and pixel intensity. (D) By measuring the protoplast diameter before lysis and the number of chloroplasts liberated after lysis a correlation is seen between both plant species, this relationship can be defined by an identical linear regression where the y-intercept is set to 0 and the slope is ($b_{yx}=2.5$). The black dashed line represents ten chloroplasts, it was recognized previously that newly created plant cells contain on average ten Proplastids, (E) which can develop into chloroplasts throughout plant development. (F) The distribution for the number of chloroplasts per protoplasts can be best fit to a gamma distribution, which shows similarity towards chlorophyll autofluorescence measurements per cell acquired by flow cytometry in 1987 by Galbraith et al.[258] (inset F).

2.8 Proof of Concept Experiments

The combination of materials and methods detailed above actualise the novel workflow for protein measurements in single protoplasts, a visual description of this can be seen in *Figure 2.17*. Specifically, microfluidic devices were fabricated following the details in section 2.2. Antibody assays were developed following the procedures in section 2.3. These two components were aligned to fabricate the MAC chip. Protoplasts were isolated from plant leaves following the directions in section 2.6. Followed by integration and lysis within the MAC chip detailed in the procedures of section 2.7. Protein binding events after protoplast lysis, were imaged and quantified through the section 2.4 technology and methodology.

To assess the MAC chip performance for protein quantitation in plants two preliminary experiments were undertaken. The first preliminary experiment was a control

experiment, to quantify the amount of autofluorescence signal generated in the antibody assay after the lysis of single protoplasts. The second, a proof of concept experiment, was performed to assess the precision of the MAC chip methodology through the single cell quantification of GFP expressed within transgenic *Arabidopsis thaliana*, explicitly a 35S: GFP cell line. This assessment was performed through a comparison of epi-fluorescent quantification methods with that of the MAC chip bi-molecular antibody assay capabilities. A technique previously explored for proof of concept experiments in mammalian cells [78].

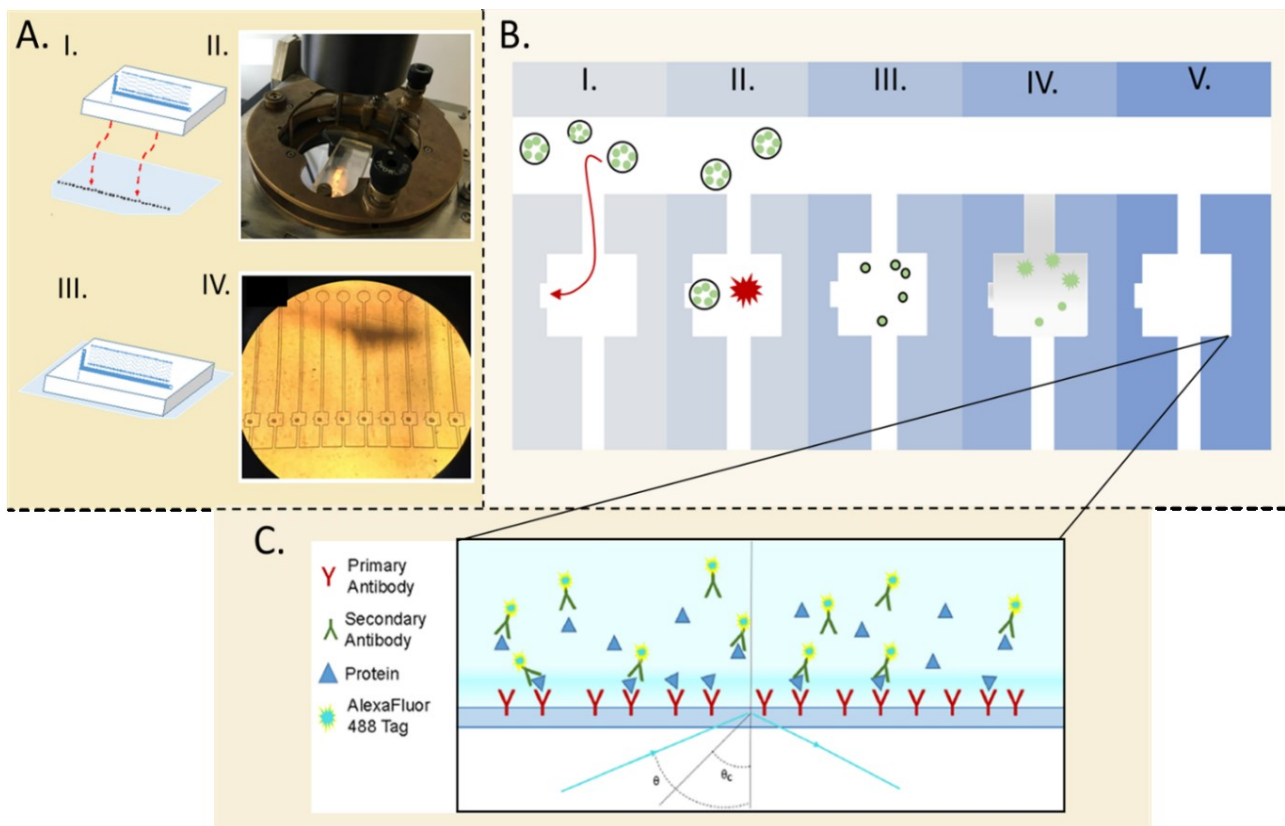


Figure 2.15 Workflow for Protein Measurements in Single Protoplasts. (A) The microfluidic device is composed of two components a PDMS mold of channels and a coverslip with printed antibody spots. These two parts are assembled (I) by implementation of a 6-axis alignment jig (II) facilitating the creation of the device titled The MAC Chip (III) each microfluidic chamber contains one printed antibody spot (IV). *Images in section A courtesy of Vanessa Ho.* After assembly pre-experimental steps are taken for antibody spot assessment, followed by the experimental workflow. (B) Protoplasts are drawn into the main channel of the microfluidic device. Through implementation of a 1070nm ytterbium fiber laser these single protoplasts are optically trapped and positioned in individual assay chambers (I.). Protoplasts undergo lysis by the introduction of an optically produced cavitation bubble (II.) releasing chloroplasts (III.) which undergo chemical lysis (IV.). Lysis facilitates the liberation of proteins for identification (V). (C) Protein identification is achieved by a tri-molecular miniaturized ELISA assay, previously explained in Section 2.3.

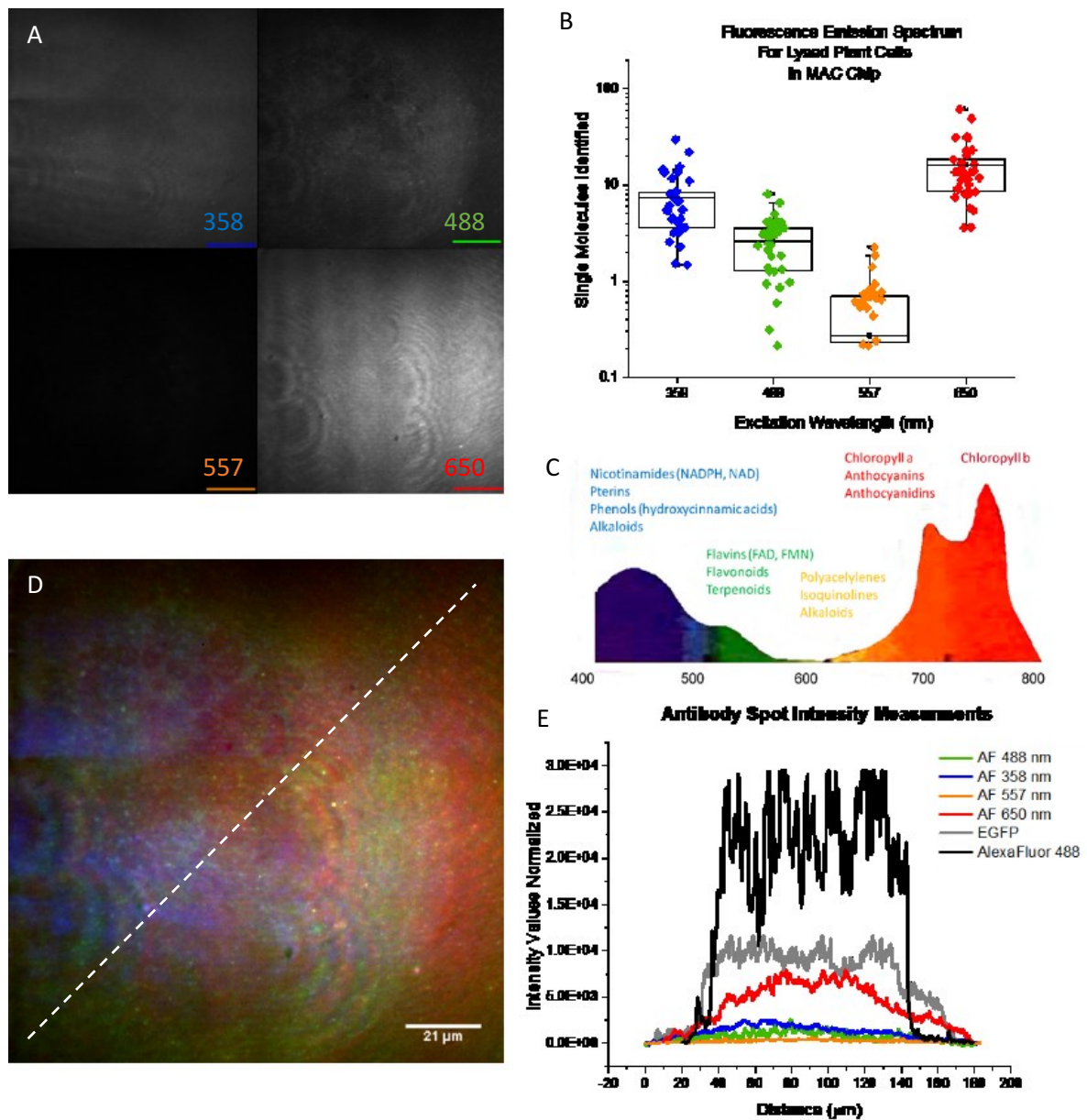


Figure 2.16 Quantification of Protoplast Autofluorescence. Preliminary experiments were undertaken to determine the amount of autofluorescence noise within the measured signal for protein quantification assays. (A) Single cells were lysed by optical then chemical methods in an analysis chamber with an antibody spot present and individual images were acquired after 1 hour by illumination from an excitation laser set at 358, 488, 557 and 650 nm. (B) These images were analyzed and quantified for the presence of single molecule and present a fluorescence emission spectrum similar to that of a leaf (C), adapted from [148]. The intensity profile for each excitation wavelength on an antibody spot was measured following the white dotted line drawn upon the wavelength compiled antibody spot (D) and the intensity profile for each wavelength was graphed and compared with non-autofluorescence measurements involving a bimolecular (GFP) or tri-molecular assay (Secondary Antibody conjugated to AlexaFluor 488) showing the measured signal is far greater than the autofluorescence emission signal when excited by a 488 nm laser line (E).

2.8.1 Autofluorescence Quantification after Lysis

Plants have unique challenges concerning fluorescent imaging, due to the autofluorescence which stems from biomolecules, such as chlorophyll, carotene and xanthophyll found within plant cells [148]. A diagram showing the fluorescence emission signature of plant biomolecules can be seen in *Figure 2.16 C*. Previous experimental evidence has shown that Alexa Fluor 488, with an excitation at 495 nm and emission at 519 nm can be utilized within plants without autofluorescence background problems. This is due to the level of quantum efficiency (ϕ) of the fluorophores, as presented in *Equation 1.1*.

Characterization of the autofluorescence in our system was achieved following the workflow presented in *Figure 2.17*; single mesophyll cells were lysed in an assay chamber containing no secondary antibody, but with a printed capture antibody spot. Images of the antibody spots were illuminated at an excitation wavelength of 358, 488, 557, and 650 nm and captured images were quantified for fluorescent binding events. Through the implementation of TIRF methodology, which only provides an evanescent field ~ 150 nm above the coverslip surface, described in section 2.4.2, and in combination with our single molecule counting algorithm, described in section 2.4.3. We characterise the autofluorescence within our system to be quantitatively minimal, raw data from one analysis chamber is shown in *Figure 2.16 A*, while the quantitative output of all measurements is shown in *Figure 2.18 B*.

2.8.2 CaMV 35S: GFP

In the second preliminary experiment expression of GFP within mesophyll protoplasts of *Arabidopsis thaliana* is driven by the addition of a widely used promoter sequence from the Cauliflower Mosaic Virus (CaMV 35S). This promoter sequence has previously been demonstrated as a strong constitutive promoter producing high levels of gene expression [149].

2.8.3 Correlation between Epi-fluorescence and MAC Chip Antibody Assay

Following the methodology outlined in *Figure 2.17*, we perform a precision assessment on this plant protein quantification method. Precision measurements are best done by assessing the correlation between a known method with a test method. In this circumstance, the known method was widefield fluorescence microscopy, to measure the amount of GFP expressed in mesophyll protoplasts. While the test method was the bi-molecular antibody assay, implemented for GFP pull down quantification. A technique previously explored for proof of

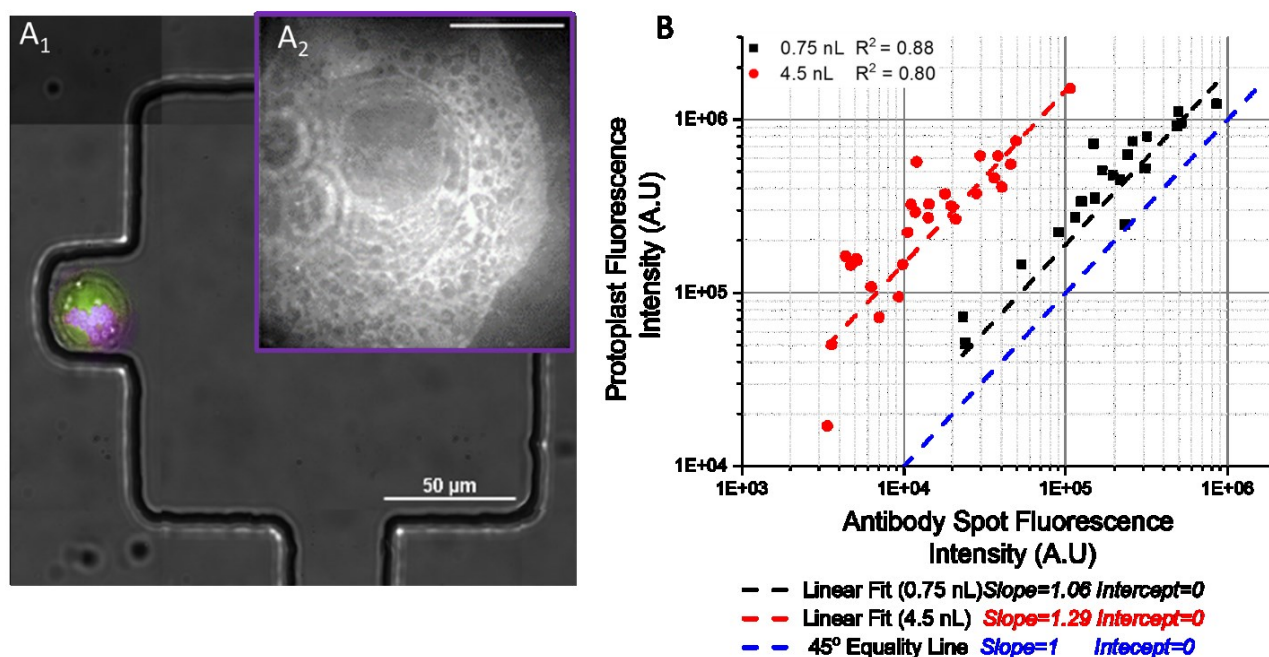


Figure 2.17 Single Protoplast 35S: GFP Fluorescence Intensity Measurements. To assess the precision of this novel plant quantitative plant biology platform. (A₁) Widefield fluorescence images were taken before the event of cellular lysis; this protoplast fluorescence intensity was directly compared to the post-lysis fluorescence intensity of the (A₂) bimolecular GFP pull-down assay implemented within the microfluidic device. (B) Presenting the experimental findings that a reduction in chamber volume from 4.5 nL to 0.75 nL increased the total antigen capture by 52% while increasing the correlation between the two fluorescence measurements methods from ($R^2 = 0.80$) to ($R^2 = 0.88$). Furthermore, the linear regression lines with a y-intercept of 0 yields a straight line with a slope of 1.06 and 1.29 for the 0.75 nL and 4.5 nL chambers, respectively. This nonsignificant deviation from the 45° equality line (slope= 1 and y-intercept= 0), highlights further evidence of measurement technology agreement

concept experiments in mammalian cells [78]. It follows from *Equation 1.7 B* that the greater the number of GFP proteins in a protoplast the higher the fluorescence intensity, measured by widefield fluorescence microscopy. These measurements of protoplast fluorescence intensity, as shown in *Figure 2.19 A₁*, were acquired prior to cellular lysis. After cellular lysis, the GFP pulldown antibody assay was quantified by images of the antibody spot after the assay reaches equilibrium, as shown in *Figure 2.19 A₂*. This assay had previously been shown to reach equilibrium after 60 minutes, as presented in *Figure 2.6 A*.

The agreement between the two methods is assessed by plotting this known method data against the test method data respective to each cell. Experiments were carried out two times, once in a MAC chip composed of 4.5 nL assay chambers and another time in 0.75 nL assay chambers, allowing the additional assessment towards volumetric alterations facilitating an increase in capture efficiency. This is predictive of *Equation 2.9*, where an increase in antibody concentration (A) would enable a higher concentration of antibody and antigen interactions (AP), generating a higher fraction of antigen bound (F_{bound}). We provide

experimental evidence, as shown in *Figure 2.19 B*, which agrees with this prediction. Reducing the chamber volume from 4.5 nL to 0.75 nL provides an increase in total antigen (GFP) capture by 52%. This assessment was made by comparison of antibody spot fluorescence intensity for cells with similar protoplast fluorescence intensity. Assessing the agreement between the two methods, a linear regression line has been plotted respective of which chamber size was implemented for each experiment. These linear regression lines were fit to the data and a high degree of correlation measured as $R^2 = 0.88$ and $R^2 = 0.80$ for 0.75 nL and 4.5 nL chambers respectively, presents evidence that the antibody assay captures a proportional level of protein which was measured by widefield microscopy.

Furthermore, these linear regression lines with a y-intercept of 0 yields a straight line with a slope of 1.06 and 1.29 for the 0.75 nL and 4.5 nL chambers, respectively. This nonsignificant deviation from the equality line represented as slope= 1 and y-intercept= 0, presenting further evidence of method agreement. In conclusion, this proof of concept assessment provides representation that the plant protein quantification method can faithfully reproduce quantitative measurements with similar precision to widefield epifluorescence measurements.

2.9 Conclusion

In summary, we have provided evidence that single plant cell proteomic measurements can be attainable, for the first time, through the utilisation of a microfluidic device titled the MAC chip. The MAC chip contains microfluidic features that are used as analysis chambers; these were optimised to limit protein diffusion while limiting chamber to chamber contamination for single cell analysis. The introduction of single plant cells is attainable through isolation methods that produce intact and photosynthetically viable protoplasts or chloroplasts for further analysis. After single plant cells have been further secluded and lysed, within individual assay chambers through optical methods, protein quantification is made possible by an antibody assay.

The microfluidic device can be altered with respect to the assay chamber volume to enhance the capture efficiency of the antibody assay used for protein quantification. Furthermore, the capture antibody or secondary detection antibody can be printed or conjugated, respectively, in different conditions to enhance assay sensitivity. We have provided evidence towards homogenous antibody spots seen in *Figure 2.5*, limiting experimental error from altered protein capture arising from altered spot morphology. The

capability of single molecule detection and quantification is achievable through TIRF microscopy and fluorophore identification as 2D Gaussian peaks within antibody spot images presented in *Figure 2.8*.

As a proof of concept, we have shown the ability to perform these methods upon *Arabidopsis thaliana* mesophyll cells, which have been modified to over-express GFP. To assess the precision towards these antibody assay measurements, epi-fluorescence measurements for the same cells were also obtained. The comparison of these two GFP intensity measurements on a single cell basis showed a very high correlation shown in *Figure 2.19*, providing evidence that this microfluidic device can be used to capture proteins from single plant cells. Additionally, we have shown experimental evidence towards the miniaturisation of chamber volume and the increase of capture efficiency for a bi-molecular antibody assay. However, for this technique to be appreciated and advantageous for the field of plant biology, it is essential that proteins are detected in their native states. Therefore, the implementation of a tri-molecular assay for protein detection will be necessary as only a handful of native proteins expressed within protoplasts have fluorescent properties.

Chapter 3

Single Cell to Organelle Rubisco

Quantification

This experimental chapter describes the optimisation of a novel tri-molecular assay for the absolute quantification of Rubisco. The assay is integrated into sub nanoliter chambers permitting single plant cell and chloroplast Rubisco measurements, discussed in context with plant cell and chloroplast development. The Rubisco tri-molecular assay is further transformed into a multiplexed assay for exploring the protein-protein interactions of Rubisco and Rubisco Activase.

3.1 Introduction

Rubisco was acknowledged as the most abundant protein on earth in 1979 and re-confirmed in 2013 with additional knowledge of Rubisco abundance in cyanobacteria and microalgae [150]. This abundance is not only exhibited on earth, in part to the high biomass of photosynthetically active organisms in the world but also within a leaf where Rubisco constitutes 50% of the soluble leaf protein [151]. This abundance within leaves has been recognised as a compensatory mechanism, as Rubisco suffers from a slow catalytic rate due to poor substrate specificity. Another mechanism to overcome Rubisco inhibition is a protein-protein interaction with Rubisco activase (RCA), whereby RCA implements ATP hydrolysis to release the inhibitor complexes within Rubisco [152].

In higher plants, this 560 kDa protein displayed in *Figure 3.1* is composed of eight small subunits (15 kDa) and eight large subunits (55 kDa) and resides within the chloroplast stroma and chloroplast stromules of healthy mesophyll cells [153,155]. Due to a single endosymbiosis event, where free-living cyanobacterium became an endosymbiont in a eukaryotic host cell, the synthesis of the Rubisco holoenzyme became a stoichiometrically coordinated process. This coordination arose as a significant amount of the endosymbiont genome was transferred to the eukaryotic nuclear genome, Ribulose biphosphate carboxylase small chain (*rbcS*) is one of those genes [146]. While the Ribulose biphosphate

carboxylase large chain (*rbcL*) gene remains within the plastid. The cellular coordination of assembling this holoenzyme is exhibited by mRNA translation of *rbcS* in the cytoplasm and subsequent transport of the *rbcS* subunit into the chloroplast. The *rbcS* subunit is then assembled within the chloroplast with the plastid synthesised *rbcL* subunit [155]. Bulk studies upon the chloroplast have shown that mRNA stability influences plastid transcriptional rates, whereby this plastid mRNA stability provides a translational level of coordinated Rubisco expression with the nuclear genome [154,156].

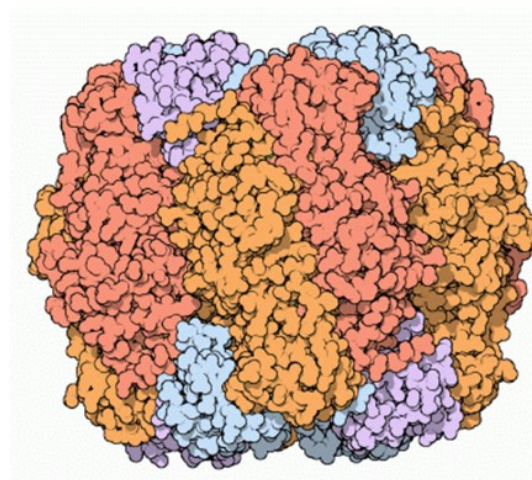


Figure 3.1 Ribulose biphosphate carboxylase/oxygenase. A cartoon depiction of Rubisco. Composed of eight RBCL that form four catalytic dimers (orange/red) and eight small subunits (blue/purple). Reproduced from reference [153]

The inefficiencies of Rubisco has generated a high amount of scientific interest, as it is a rate-limiting step for maximum photosynthetic efficiency during conditions of limited CO_2 and saturated irradiance [157]. This interest occurs as Rubisco is the initial enzyme in both photosynthetic and photorespiratory metabolism as it catalyses the carboxylation of ribulose-1,5 biphosphate and the oxygenation of ribulose-1,5 biphosphate respectively [158]. Through the development of a miniaturised ELISA assay for Rubisco quantification and its incorporation into the workflow outlined in section 2.8, this chapter will be devoted to adding insight to this scientific pool of information. Specifically, we have conducted measurements of Rubisco expression throughout plant cell development at the single cell and organelle resolution.

3.2 How Much Rubisco in the Average C3 Cell?

Before creating a protein detection assay, it would be beneficial to calculate the average Rubisco content within a single cell, creating a target for the linear regime of the detection assay. It has been shown from previously published bulk analysis methods that Rubisco constitutes up to 50% of total soluble protein within chloroplasts. Therefore, Rubisco represents 200 mg/mL of the total chloroplast protein content which has been estimated to be 400 mg/mL with the volume of a chloroplast being 3×10^{-5} nL [159].

$$\frac{\text{mass concentration (g L}^{-1}\text{)}}{\text{molar mass (g mol}^{-1}\text{)}} \times [\text{Avogadro's Constant (6.023E}^{23}\text{mol}^{-1}\text{)}] = \frac{\text{molecules}}{L} \quad (3.1)$$

$$\frac{\text{molecules}}{L} \times \text{Chloroplast Volume (3.3E}^{-14}\text{)} = \text{protein molecules per chloroplast} \quad (3.2)$$

Imputing from these measurements into *Equation 3.1* and *3.2*, it can be estimated that 7×10^6 molecules of Rubisco exist within the average chloroplast. By knowing the average number of chloroplasts within a protoplast from the previous measurements obtained in section 2.7.4, we can further estimate the average Rubisco content within a protoplast to be 2×10^8 molecules of Rubisco. In conclusion, an ideal Rubisco detection assay would show a linearity response for three orders of magnitude around these two estimated Rubisco contents.

3.3 Rubisco Calibration Curve

The ability to convert the antibody spot fluorescent intensity measurements into absolute protein copy numbers is accomplished through a calibration curve. Calibration curves were achieved by the introduction of a solution containing a known concentration of protein vacuum filled into the MAC chip analysis chambers. The linear region of the calibration curve is identified and used for comparison towards the single cell experimental single molecules counted, facilitating the quantification of absolute protein copy number within analysis chamber from the experimental antibody spot images.

3.3.1 Methods Applied for Rubisco Calibration Curve

The Rubisco implemented for assay selection, and calibration was donated by Kerry O'Donnelly. Rubisco was isolated from Spinach oleracea by employing hydrophobic interaction chromatography and resuspended to 10 μM in (50mM Tris-HCl, 20mM MgCl_2 , 20mM NaHCO_3 , 0.2mM EDTA and 2mM DTT brought by the addition of KOH to a pH of 7.6) [160]. Bradford Assays for protein concentration measurements were performed by mixing 20 μL of protein solution with 300 μL of Bradford Reagent (Sigma Aldrich, UK) incubated for 5 minutes and analysed by a spectrophotometer for absorbance at 595 nm. This measurement is based upon the proportionality between the amount of light absorption and the amount of protein present in the solution. A relationship defined by the Beer-Lambert law, previously presented as Equation 1.4. To determine the purity of a protein, samples were run on SDS-PAGE gels as described in section 2.3.5. After the proteins were separated on the

gels they were stained for 20 minutes with a solution of Coomassie Blue R-250 (10% Acetic Acid, 50% Methanol and 0.1% Coomassie Blue R-250 (v/v)) followed by overnight incubation in a de-staining solution (40% Methanol and 10% Acetic Acid (v/v)).

Preliminary antibody qualification experiments were performed by demarcating a hydrophobic barrier using a wax pen around the printed capture antibodies providing an area to contain a 30 μL solution. Within this solution resides 1×10^7 secondary antibody molecules per nL and 1×10^6 Rubisco molecules per nL. After antibody combinations have been trialled and chosen, the process of measurements for calibration curves is performed. By introducing a solution (4% BSA (w/v), W5, and 1×10^7 molecules/nL secondary antibody) spiked with a known Rubisco content of $1 \times 10^3 - 10^9$ molecules/nL calculated by Equation 3.1 into a MAC chip with 45 printed capture antibody spots, one spot per assay chamber. Single molecule counts for each antibody spot was performed by implementing the methods outlined in 2.4.3.

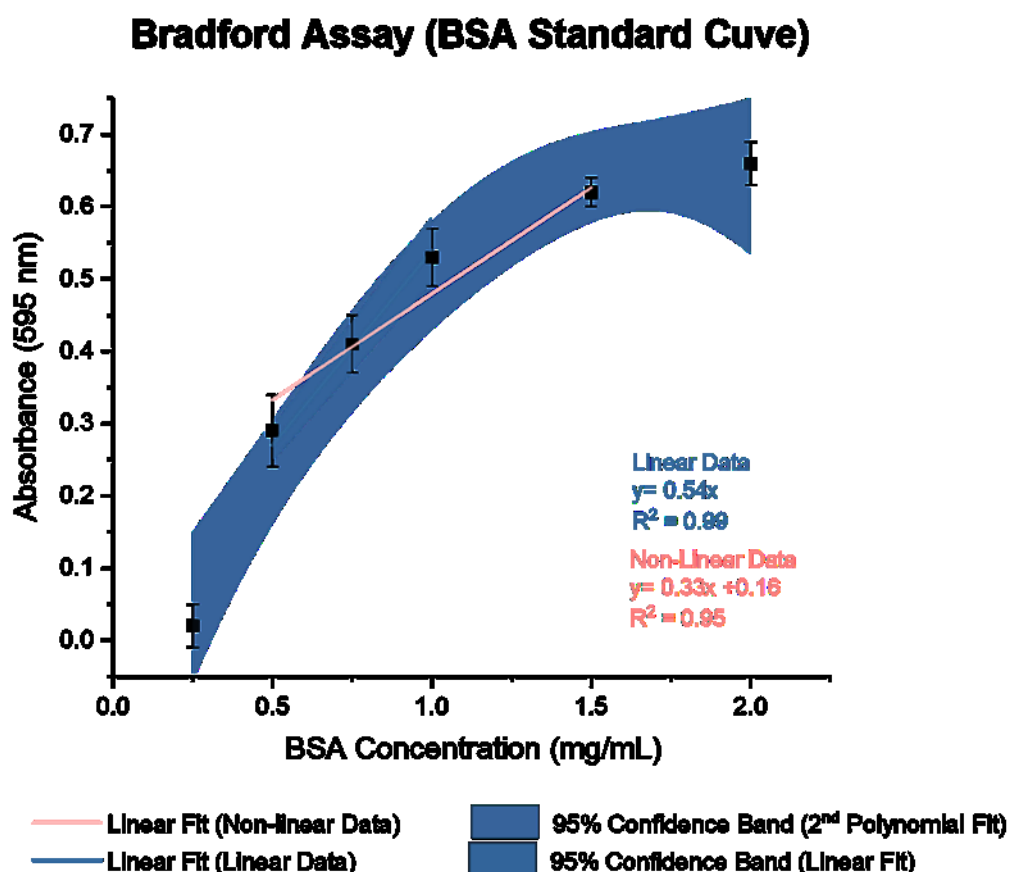


Figure 3.2 BSA Standard Curve. To quantify unknown concentrations of Rubisco a Bradford assay was implemented. Calibration of the assay was performed using known concentrations of BSA, resulting in a standard curve exhibiting a polynomial non-linear fit. This polynomial non-linear fit with a known concentration of 0.5-1.5 mg/mL can be estimated by a linear fit of ($y = 0.33x + 0.16$). While the true assay linear region between 0.5-1 mg/mL can be fit to the equation ($y = 0.54x$).

3.3.2 Characterization of Isolated Rubisco

The protein introduced into calibration curves can either be a recombinant protein, that is protein manufactured in an alternative cell-types in a high-throughput mechanism, or the protein can be purified directly from the organism. The latter method was chosen for the Rubisco implemented within these calibration experiments and had been directly purified from *S. oleracea*. The concentration of provided Rubisco was calculated by implementing a Bradford Assay following the manufactures instructions for the detection of 1-1,400 µg/mL protein as detailed in section 3.3.1. First, a standard curve using known concentrations of BSA was created as shown in *Figure 3.2*. Next, a sample of the provided Rubisco was diluted 3x and was measured as an absorbance of 0.639 at 595nm, this equates to ~1.5 mg/mL, and the provided sample would be interpreted as 4.5 mg/mL. However, as the absorbance measurement for this 3x diluted Rubisco sample falls outside the linear region of the BSA standard curve, the accuracy of this measurement is questionable. Therefore, a 6x diluted sample was measured presenting an absorbance value of 0.455 at 595 nm, this equates to ~0.85 mg/mL for the 6x diluted sample and highlights the importance of only performing quantification measurements in the calibrated linear range. From this final Bradford assay measurement, the total protein concentration provided was estimated to be 5.1 mg/mL.

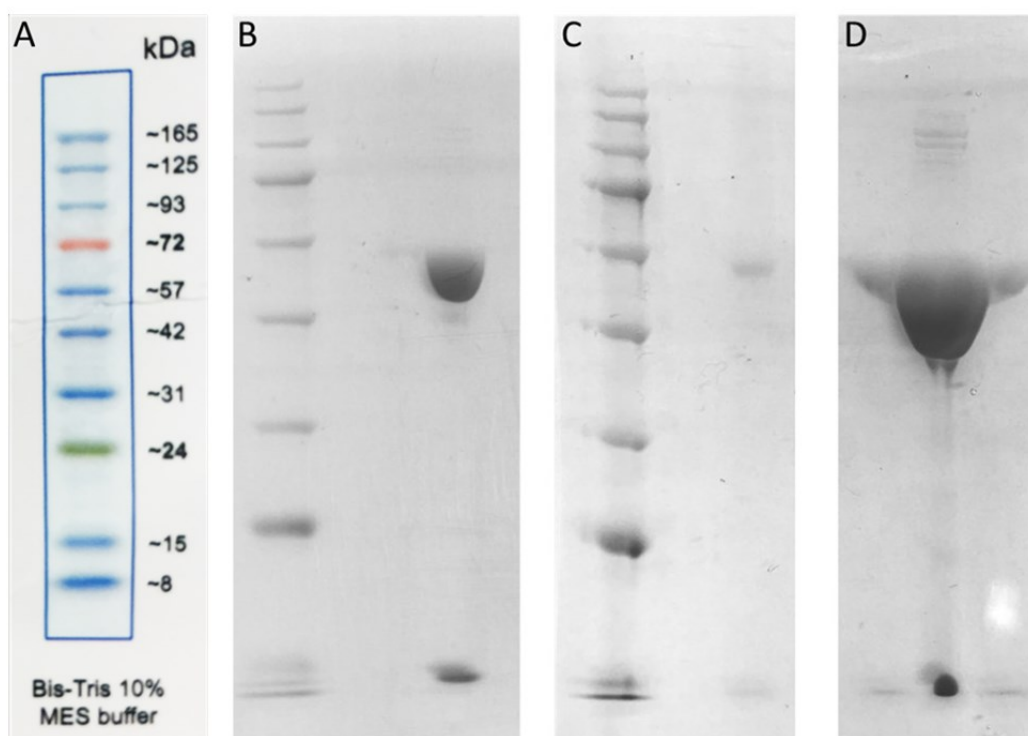


Figure 3.3 Purity Analysis of Rubisco. To assess the purity of the Rubisco sample provided, two polyacrylamide gel electrophoresis experiments were performed, (A) using a protein ladder to demarcate protein weight. (B) The first gel contained a 3x diluted sample of Rubisco displaying both large and small subunits (88%). With a slightly pronounced (12%) band at 41 kDa, this band is representative of the smaller Rubisco Activase isoform (a protein shown to associate with Rubisco). A second gel was performed with a (C) 6x diluted sample and (D) no dilution of the sample.

To determine the purity of the Rubisco sample 5 μ L dilution series samples were ran (i.e. 6x 3x and 0x dilution) on SDS-Page following the protocol outlined in section 2.3.5. It was previously identified that hydrophobic interaction chromatography can produce Rubisco samples from *Spinacia oleracea* with a purity > 90%. As can be seen in *Figure 3.3* after the Gel was stained, the sample provided which underwent 3x dilution was of 88% purity, determined from densitometric analysis, displaying two prominent bands at ~55 kDa and ~15kDa, representative of the large and small subunit of Rubisco respectively.

3.3.3 Rubisco Capture Antibody

As previously discussed, the alteration from a bi-molecular to a tri-molecular assay involves the need for a detection antibody. However, before possible detection antibodies were tested, the affinity purified polyclonal capture antibody (Lot Number: 1501; Item No. AS03 037A; Agrisera, Switzerland) was chosen. The capture antibody was deemed proficient in affinity and specificity for use in the Rubisco assay due to the manufacture's validation of Rubisco detection from 31.25 femtomoles. Furthermore, this antibody was shown to have a high-specificity towards Rubisco as shown by a western blot in *Figure 3.4*. Performed by Dr. Malgorzata Wessels, for a 10 μ g total protein sample from *Arabidopsis thaliana*. Another benefit of this antibody was the format in which it was provided; lyophilised in PBS, with no additives, a notable characteristic for high-performance capture antibodies due to adulteration from additives promoting poor spot homogeneity.

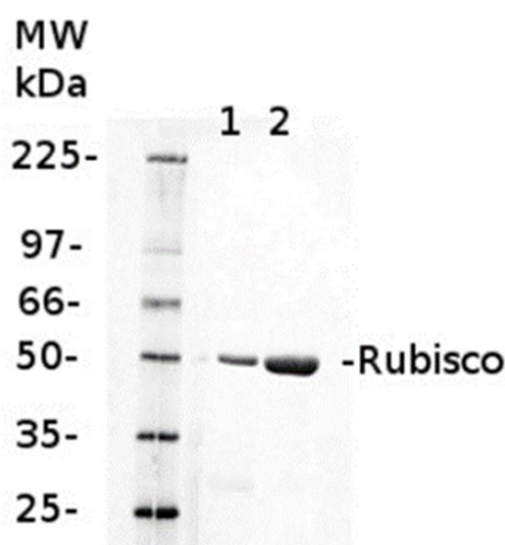


Figure 3.4 Rubisco Capture Antibody WB. Western Blot was performed on 1 μ g of chlorophyll (1) or 10 μ g of total protein from *Arabidopsis Thaliana* leaves (2). Anti-Rabbit DyLight 550 secondary antibody was implemented to visually depict interactions of Rubisco with rabbit anti-Rubisco AS03 037A. Image Courtesy of Dr. Malgorzata Wessels

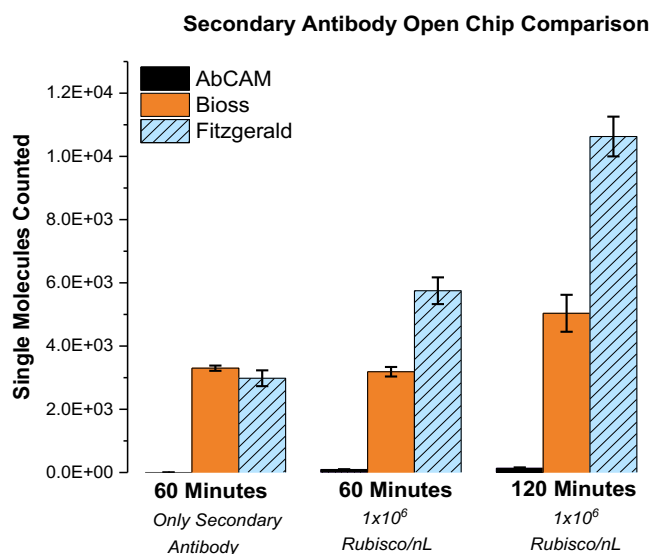


Figure 3.5 Rubisco Secondary Antibody Comparison. Through single molecule measurements in open chip experiments the Fitzgerald secondary antibody provided the greatest signal. The first 60 minutes are NSB interactions between the secondary antibody and capture antibody followed by binding interactions for 120 minutes with 1×10^6 Rubisco/nL.

3.3.4 Rubisco Secondary Antibody

Three different secondary antibodies were compared for performance by an open chip microarray. The first detection antibody was acquired from Bioss, purified by Protein A, raised against a KLH conjugated synthetic peptide derived from *Arabidopsis thaliana* and conjugated to AlexaFluor 488 (Item No. bs-6988R-A488; Bioss, USA). The second detection antibody was purchased from Abcam, it was raised against full-length Rubisco Small Subunit (Q9TIM3) and was conjugated to AlexaFluor 488 (Item No. ab62391; Abcam, USA) by applying a conjugation reaction. The conjugation reaction used the Lightning-Link® Rapid Conjugation System and was carried out as specified by manufacturer instructions (InnovaBiosciences, UK). The third detection antibody trialled was obtained from 2B Scientific, the antibody was raised using Rubisco purified from *Spinacia oleracea* as the immunogen, purified by ammonium sulfate precipitation and conjugated with FITC. As can be seen in *Figure 3.5*, the Fitzgerald antibody (Lot Number: CR78798 Item No. 60R-2258; 2B Scientific, UK) outperformed the other antibodies with respect to generating a higher signal intensity upon the addition of 1×10^6 Rubisco molecules per nL; this was measured by the methods presented in Section 2.4, and resulted in choosing Fitzgerald secondary antibody for the integration into the Rubisco assay.

3.3.5 Western Blot Validation

Western blots, following the methods outlined in section 2.3.5, were performed to validate the specificity of the chosen secondary antibody. Rubisco detection in lysate samples using

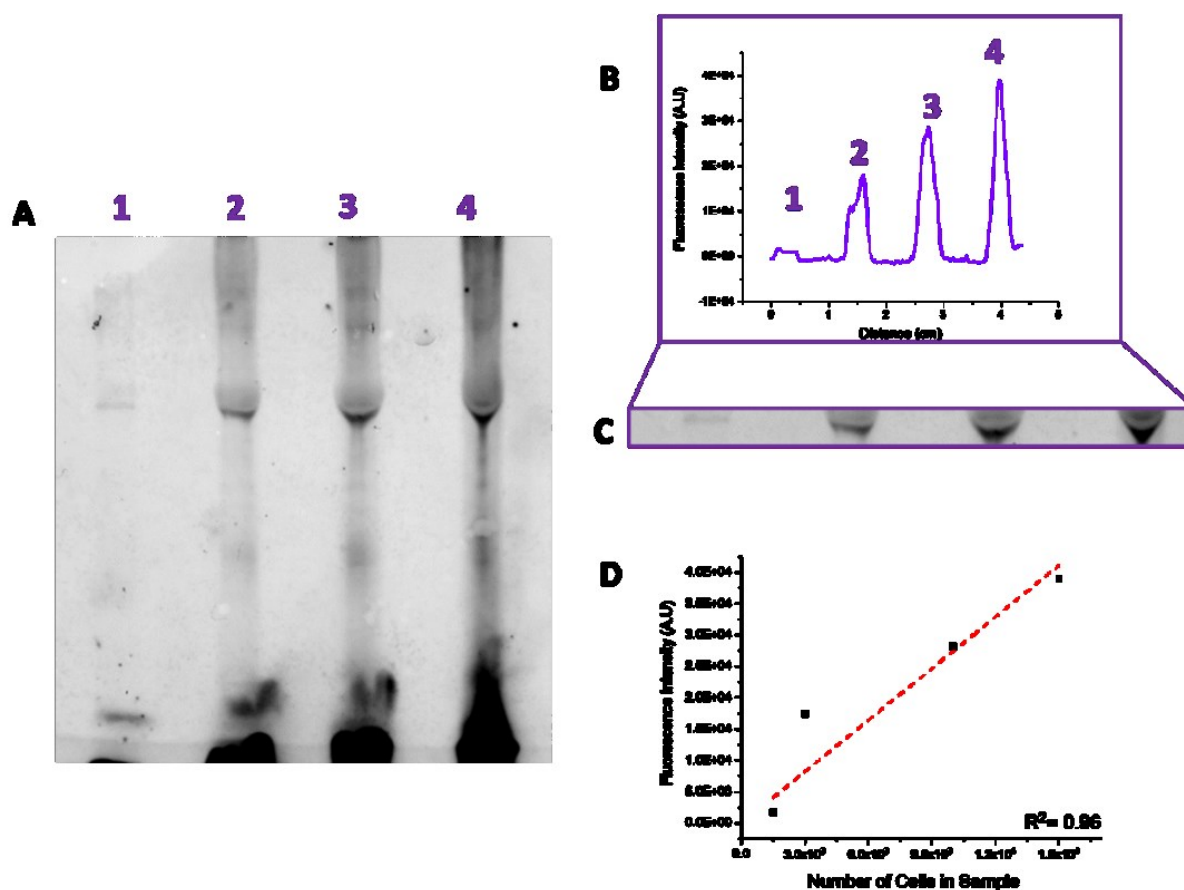


Figure 3.6 Spinach Lysate Western Blot Directly Probed with Fitzgerald Secondary Ab. To assess the affinity of the chosen Fitzgerald secondary antibody towards Rubisco (A) a western blot was performed on lysate samples. Each lane (1-4) was loaded with increasing protoplast number/sample, (B) producing relatively increasing fluorescent intensity for the identified ~55 kDa Rubisco band. (D) A high correlation is exhibited between the number of cells in the sample and the fluorescent intensity of the Rubisco band when the y-intercept is fit to zero ($R^2 = 0.96$).

the Fitzgerald antibody is displayed in *Figure 3.6*. Each lane was loaded with a known concentration ranging from 1.5×10^3 to 1.5×10^4 cells per lane. By summing the fluorescence intensity of the Rubisco bands identified, shown in *Figure 3.6 C*, and comparing this with the cell content per lane a high linear correlation ($R^2 = 0.96$) is demonstrated. Furthermore, the western blot technology, specifically in lane 1 in *Figure 3.6* where lysate from 1.5×10^3 cells has been analysed, qualitatively demonstrated the high specificity of the Fitzgerald antibody towards the Rubisco Large Subunit and Small Subunit in the cell lysate.

3.3.6 Rubisco Calibration Curve

It has previously been demonstrated in section 2.8.3 that miniaturisation of chamber volume can enhance the sensitivity of a bi-molecular assay. To understand if this effect can be witnessed in a tri-molecular assay, calibration curves for Rubisco, using the previously described Rubisco sample, capture antibody, and Fitzgerald secondary antibody was analysed in both 4.5 nL and 0.75 nL MAC chip assay chambers. The resulting linear range

presented upon the addition of $1 \times 10^5 - 1 \times 10^8$ Rubisco molecules per nL which presented fluorophore counts higher than the nonspecific background (i.e. when no difference is counted between the number of fluorophores in the chamber without Rubisco to that with Rubisco) can be described within the 4.5 nL calibration curve fit to ($y=0.49 X-0.2$) while within the 0.75 nL the linear line fit was ($y=0.73x-0.5$) as shown in Figure 3.7. The difference in slopes can be reasoned that the incubation time of 1 hour may not be sufficiently long enough for the analytes present in the 4.5 nL chamber to achieve equilibrium. The two assay volumes produced an ambient analyte assay such that the linear range in the 0.75 nL chamber captured $1.2 \pm 0.3\%$ of the Rubisco in the chamber and the 4.5 nL chamber captured $0.07\% \pm 0.016\%$.

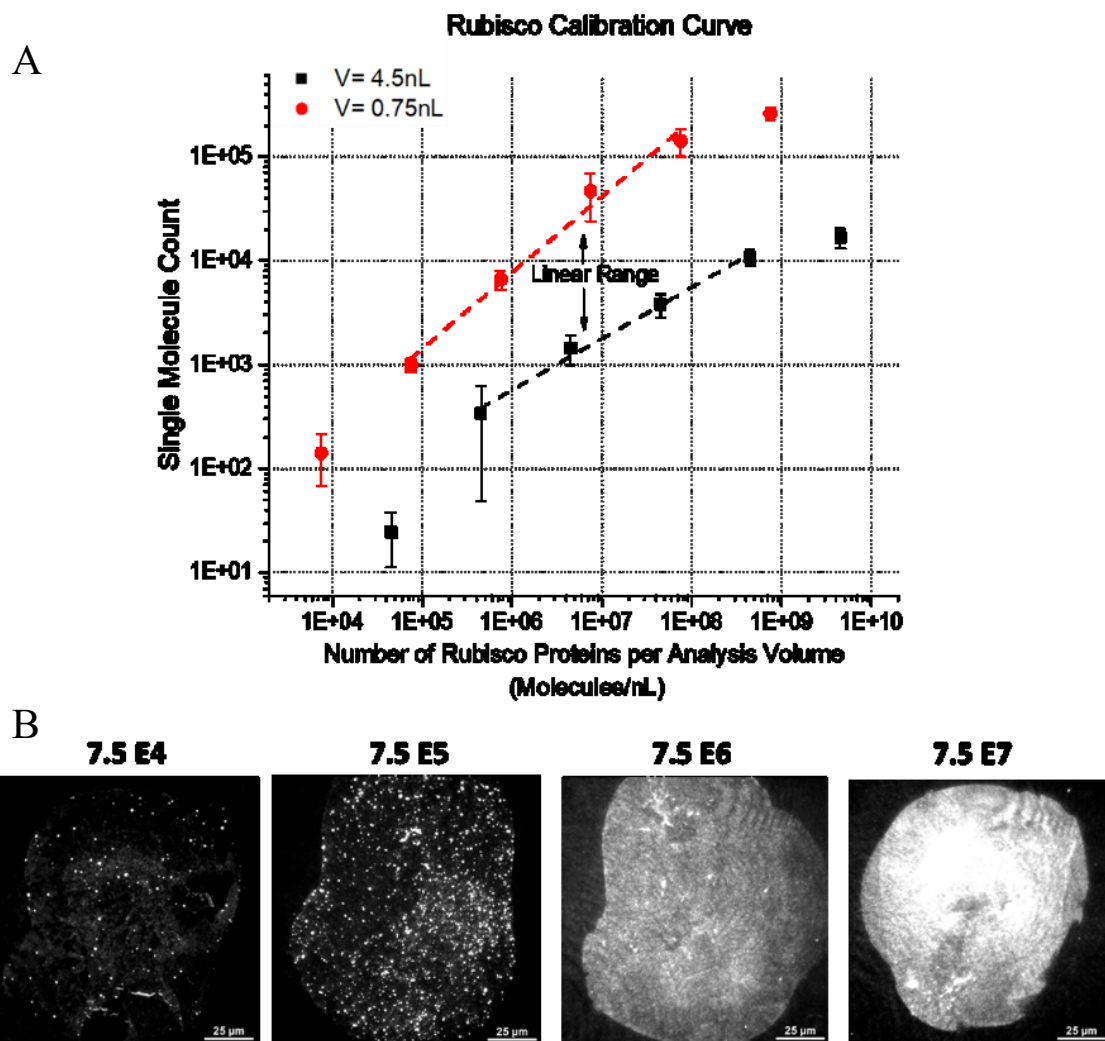


Figure 3.7 Rubisco Calibration Curve. A known amount of Rubisco is introduced into 0.75 nL and 4.5 nL microfluidic assay chambers. After equilibrium has been reached ($t=60$ minutes) the tri-molecular bound Rubisco, identified by the detection antibody is quantified as single molecule binding events, technique for quantification discussed in section 2.4.3. A linear range ($y= 0.73x-0.5$ and $y= 0.49x-0.2$ for 0.75 nL and 4.5 nL assay chambers, respectively) is identified between the Rubisco (molecules/nL) introduced into the assay and the binding events measured (single molecule count). The linear range allows a standardized Rubisco content to be recognized with only the identification of the single molecules bound at assay equilibrium. (B) Raw images of tri-molecular assay binding in the linear range from the 0.75 nL chamber Rubisco calibration, where the above number denotes the Rubisco molecules/nL present.

3.4 Single Mesophyll Protoplast Rubisco Measurements

This section presents the first use of the calibrated Rubisco assay to assess the distribution of Rubisco expression from populations of single mesophyll protoplasts isolated from Spinach oleracea grown under the customary medium-light intensity (MLI) ($\sim 350 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or transferred after plants had four true leaves into high-light intensity (HLI) ($\sim 650 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) for continued growth. The specifics of obtaining plant material has been outlined in section 2.6.1. This is followed by further measurements of Rubisco expression during leaf development from maturation until senescence. Alterations in Rubisco content during senescence occurs in response to the essential salvage and redistribution of nitrogen to newly developing leaves. Subsequent experiments upon Rubisco expression at single cell resolution provide correlated insight towards mesophyll cell development and chloroplast development with Rubisco content.

3.4.1 High Light Intensity vs Medium Light Intensity Rubisco Content

Previous evidence of alterations in bulk leaf Rubisco expression as a function of prevailing light conditions has been confirmed by western blot analysis for *G. monostachia* and rocket immunoelectrophoresis for *N. tabacum* [161,162]. This alteration in Rubisco, a main enzymatic step in CO_2 fixation in photosynthesis, has been rationalised as the leaf optimising its photosynthetic efficiency concerning the incident white light received during growth. Under MLI, the customary intensity of light applied for spinach growth, photodamage effects on leaf development are minimal allowing reproducible leaf expansion and development [163]. However, when transferred into stressful conditions such as HLI, photoprotection responses increase while photosynthetic machinery decrease in a regulated fashion.

Single-cell Rubisco expression measurements have not been performed. Therefore, we found this an ideal first test to measure the sensitivity of the calibrated Rubisco assay within 0.75 nL MAC chip chambers. Two experiments were performed following the detailed MAC chip experimental protocol provided in section 2.8. The first experiment measured Rubisco in protoplasts that were grown under MLI. These cells expressed on average $3.6 \pm 1.64 \times 10^7$ Rubisco copies. The second experiment measured the Rubisco content in protoplasts originally grown under MLI but transferred into HLI after the emergence of the third and fourth true leaf. The leaves that underwent acclimation after being exposed to HLI for ~ 20 days underwent a change in both chlorophyll content as shown in *Figure 3.8 A* and Rubisco expression producing on average $2.2 \pm 0.9 \times 10^7$ copies. This was a 0.62-fold reduction from the MLI protoplasts *Figure 3.8 B*. Data evaluation between the two measurements was

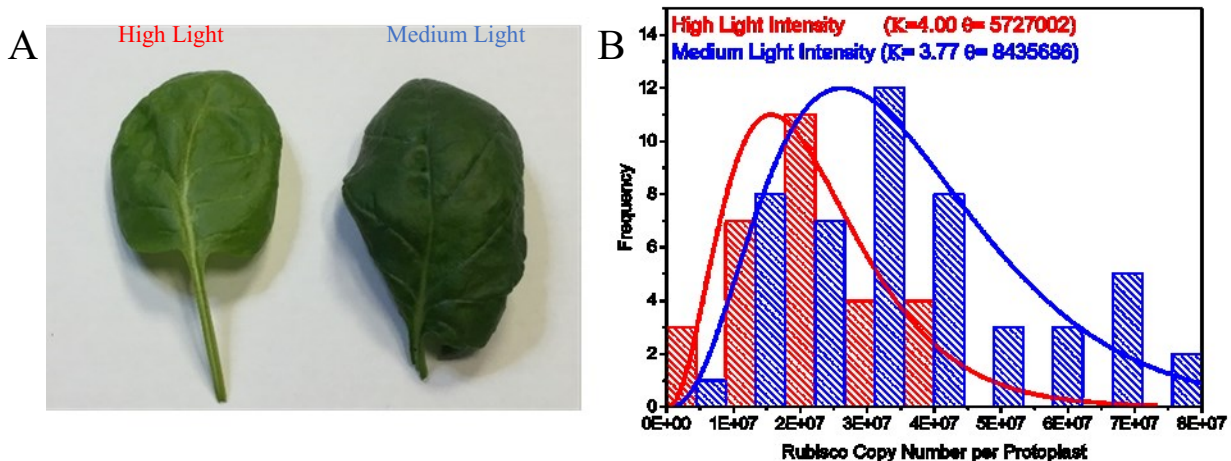


Figure 3.8 Comparison of Rubisco Expression in HLI and MLI Spinach Protoplasts. Chlorophyll expression (A) visually interpreted is altered when spinach leaves were exposed to HLI compared to MLI. (B) This change in light conditions also alters the Rubisco expression level from an average 3.6×10^7 in MLI to 2.2×10^7 for HLI. However, both distributions do exhibit a similar (K) shape parameter for Gamma distribution probability fit data.

performed by a one-way ANOVA using a Bonferroni test ($\alpha=0.05$), there is a significant difference between both measurement means. Gamma distribution parameters were measured using the methods presented in section 2.5.2.

Remarkably, under the photosynthetically high-stress environment of HLI, the measured distribution shape (variance-to-mean relation) remained similar. That is, the shape parameter characterised by the gamma PDF is $K=4.00$ for HLI and $K=3.77$ for MLI. The similarity of this parameter was surprising. As we have previously noticed that when mammalian cell gene expression is perturbed by the addition of drugs, the shape parameter either increases or decreases, specifically shown in *Figure 5.20*. This high control of distribution shape is analogous to high abundance proteins measured within *E. coli*, producing similar variance-to-mean relation under a range of varied conditions [164]. The ability for protoplasts in stressful conditions to maintain similar variance with respect to the mean value defined by the K value from the gamma distribution provides probable evidence that the regulatory mechanism of Rubisco content is continually regulated with respect to the photosynthetic environment through alterations to mRNA half-life [71]. It appears from direct measurements upon *N. tabacum* that Rubisco content in response to light conditions is maintained by alterations in the amount of nuclear-encoded SSU mRNA. While the LSU mRNA, which is encoded within the chloroplast, maintains similar quantities under changing light conditions [162].

3.4.2 Senescing Leaf Rubisco Content

To further probe the expression noise of Rubisco within leaves under modifying conditions, we chose to evaluate senescent leaves. During senescence, older leaf mesophyll cells degrade

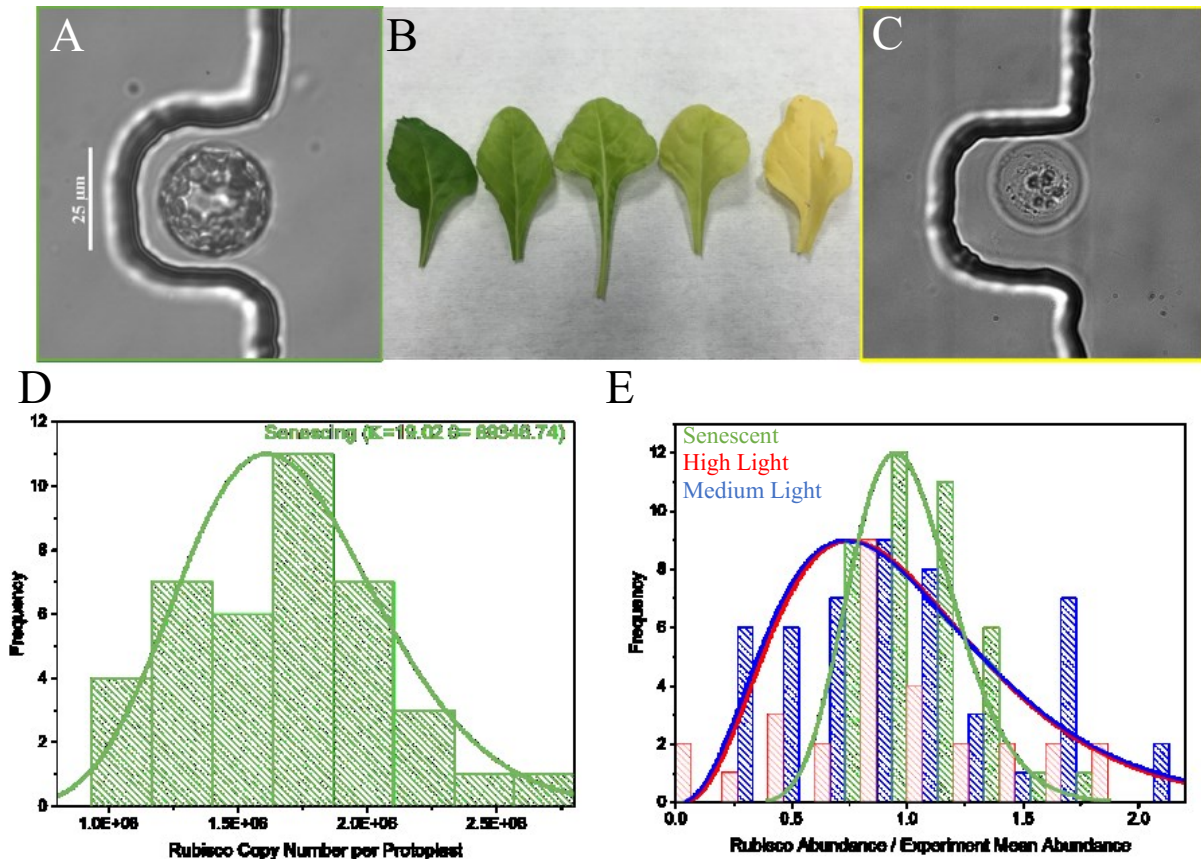


Figure 3.9 Visual and Rubisco Content Comparison between Senescent and Healthy Spinach Protoplasts. In an effort to salvage nitrogen from fully developed mesophyll cells and transfer nitrogen to newly developing leaves, the older leaves undergo senescence, which can be visualized by a color change (B). Chloroplasts containing chlorophyll and proteins, such as Rubisco, undergo degradation leaving plastoglobuli and gerontoplasts within a senescent mesophyll protoplast (C). The measured Rubisco expression within senescent protoplasts shows an alteration in the Gamma probability distribution fit shape parameter, similar to a decreased protein expression (D, E).

Rubisco to salvage and redistribute nitrogen to young and/or developing leaf tissues as Rubisco accounts for 15-30% of total leaf Nitrogen content in C3 species [165]. Through bulk measurements upon the leaf blades of *O. sativa*, it was presented that the decrease levels of Rubisco through senescence are accompanied by a decrease in both LSU and SSU mRNA.

As shown in *Figure 3.9 B*, senescent leaf tissue can be visually identified by the lack of chlorophyll and the yellow pigmentation associated with an increase in carotenoid biosynthesis [166]. Compared to the green mature or developing leaf tissue which contains higher amounts of chlorophyll. This differentiation can further be identified within single cells *Figure 3.9 C*. Senescent mesophyll cells accumulate plastoglobuli (lipid droplets) and the majority of chloroplasts have dedifferentiated into gerontoplasts [167].

Two MAC chip experiments were performed using protoplasts isolated from yellow senescing spinach leaves. The average measured Rubisco content was $1.7 \pm 0.4 \times 10^6$ copies per cell, this is a 20-fold reduction from the typically measured Rubisco content. The Rubisco expression distribution was also altered, such that the tail within the gamma PDF decreased

in length descriptive of the shape parameter $K=19.02$ as shown in *Figure 3.9 D*. This alteration in distribution is akin towards mammalian knockdown experiments, where cells are treated with siRNA or undergo drug treatment to decrease protein expression [71].

3.4.3 Rubisco Content in Relation to Cell Development

During plant leaf development, mesophyll cells migrate from regions of high mitotic division events designated as the meristematic region. Within Spinach this high mitotic division is localised to the base of the leaf during growth [163]. As mesophyll cells migrate away from this area they continue to expand, this expansion has been associated with the number of chloroplasts within the cell, which is driven by asynchronous chloroplast fission [146]. Through associating the number of chloroplasts with the mesophyll cells, we are able to construct a timeline of cell development with respect to Rubisco content.

By measuring 77 mesophyll cells, acquired from healthy spinach *Figure 3.10A*. We present a Rubisco distribution, acquired from a population of protoplasts showing a shape parameter of $K=3.54$ and an expression noise (σ^2/μ^2) of 0.28. These experiments also demonstrate an association (Pearson's $r= 0.91$) between the increase of mesophyll cell diameter and copy number of Rubisco as shown in *Figure 3.11A*. As the protoplasts undergo

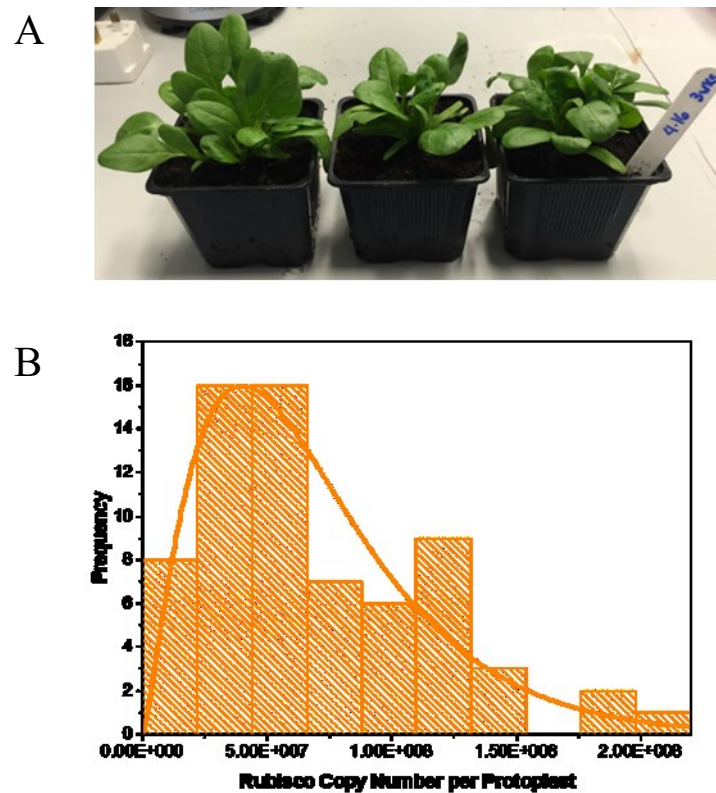


Figure 3.10 Distribution of Rubisco Expression in Mesophyll Protoplasts. (A) An exemplar image of healthy spinach plants harvested for protoplast isolation (B) The distribution of Rubisco expression, fit to a Gamma distribution probability measured from 77 protoplasts in 0.75nL MAC chip experiments shows a shape parameter of $K= 3.54$ with an average expression of $6.91 \pm 4 \times 10^7$.

development the Rubisco content increases from 10-200 million copies, this can be reasoned with previous studies that have shown that transcriptional and translational output scales with cell size. Explained as larger cells need to produce and maintain a higher amount of cellular components for sustained concentrations for optimal catalytic function [168]. The optimal Rubisco concentration can be calculated for these protoplasts, as after cell wall digestion the cells manifest as spherical bodies encapsulated by a single plasma membrane, which can be spatially quantified using the methods outlined in section 2.4.4. The maintained average concentration of Rubisco for these protoplasts is 7.69 ± 2 mM, which is progressively reached later in cell development, as shown in *Figure 3.11C*. It has previously been shown that the expression of photosynthetic genes, such as Rubisco, is highly expressed in young expanding leaves, this may account for why the concentration of Rubisco is more significant in smaller developing cells [169].

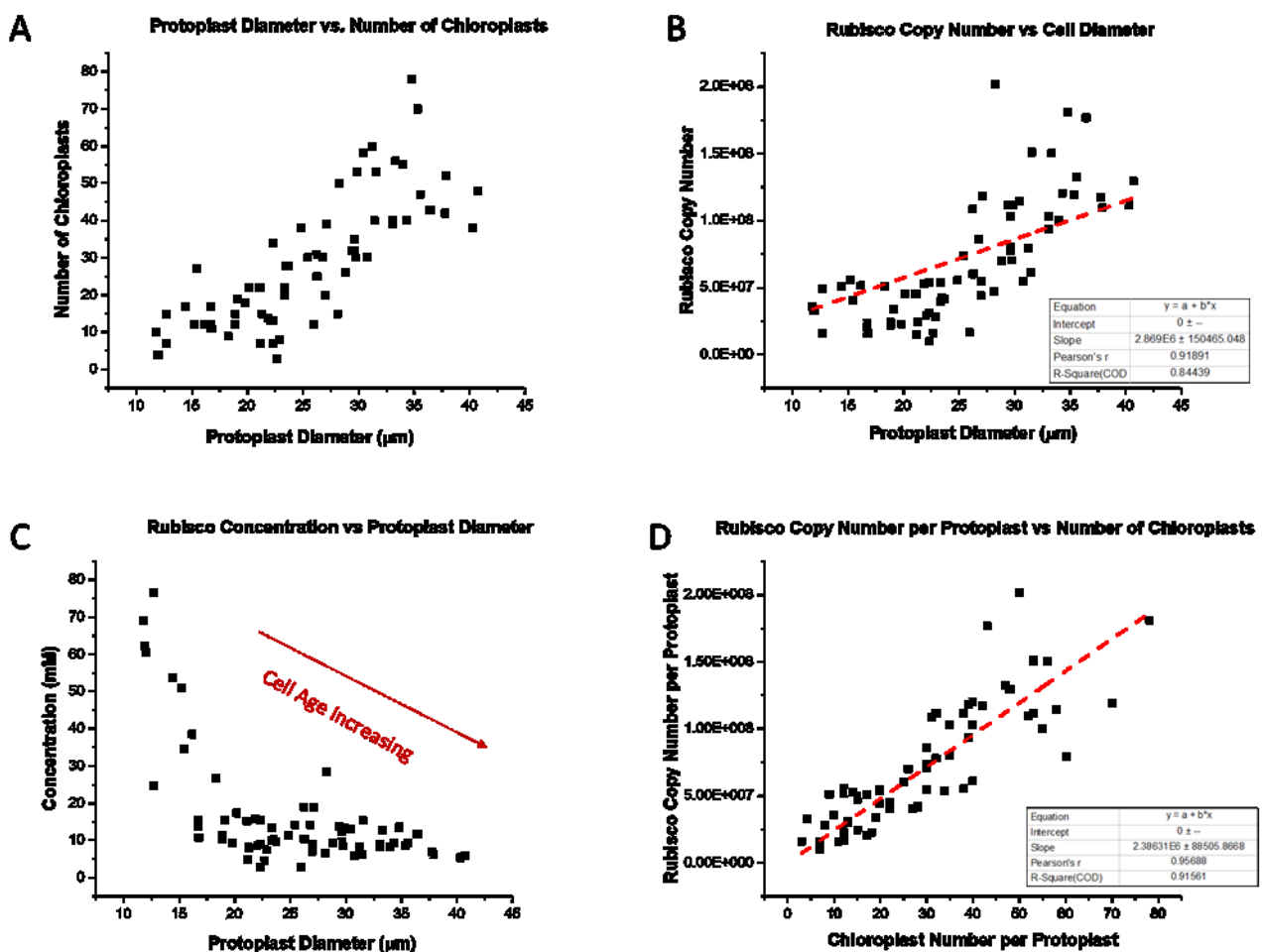


Figure 3.11 Developmental Impact on Rubisco Expression. Mesophyll cell development is (A) characterized by an increase in cell volume and the number of chloroplasts. (B) We note that as the cell develops the copy number of Rubisco increases, (C) yet through development the concentration of Rubisco decreases to a maintained level of 7.69 ± 2 mM. (D) Additionally, the highest linear correlation measured between plant development and Rubisco content is between the number of chloroplasts present in the protoplast ($R^2 = 0.91$).

Furthermore, a higher correlation of association with respect to protoplast diameter and Rubisco copy number is witnessed when the number of chloroplasts within the protoplast is compared with the measured Rubisco copy number (Pearson's $r=0.95$). Seen in *Figure 3.11 D*. As Rubisco is predominantly found within the chloroplast, and the number of chloroplasts per each protoplast is under identical nuclear control from SSU mRNA content. Therefore this higher agreement is not surprising. From this data, we can estimate the Rubisco copy number per chloroplast within each protoplast ($n=68$) as shown in *Figure 3.12A*. The data presents an average chloroplast expression of 2.7 ± 0.2 million Rubisco molecules per chloroplast, where 37% of the protoplasts measured were above this average expression, and 63% were below the average. The chloroplasts above the average Rubisco content ($n=19$) have an average cell diameter of $22.07 \pm 1.8 \mu\text{m}$ while the below average chloroplasts ($n=49$) have an average cell diameter of $26.69 \pm 1.0 \mu\text{m}$, as shown in *Figure 3.12B*. These estimated calculations provide unresolved evidence that as cells acquire more chloroplasts through development, the Rubisco copy number per chloroplast decreases, yet the copy number within the protoplast increases. Implying evidence of Rubisco expression regulation during development, by either the protoplast or chloroplast. This regulation mechanism appears to allow the protoplast to “tune” into the ideal Rubisco concentration for photosynthesis

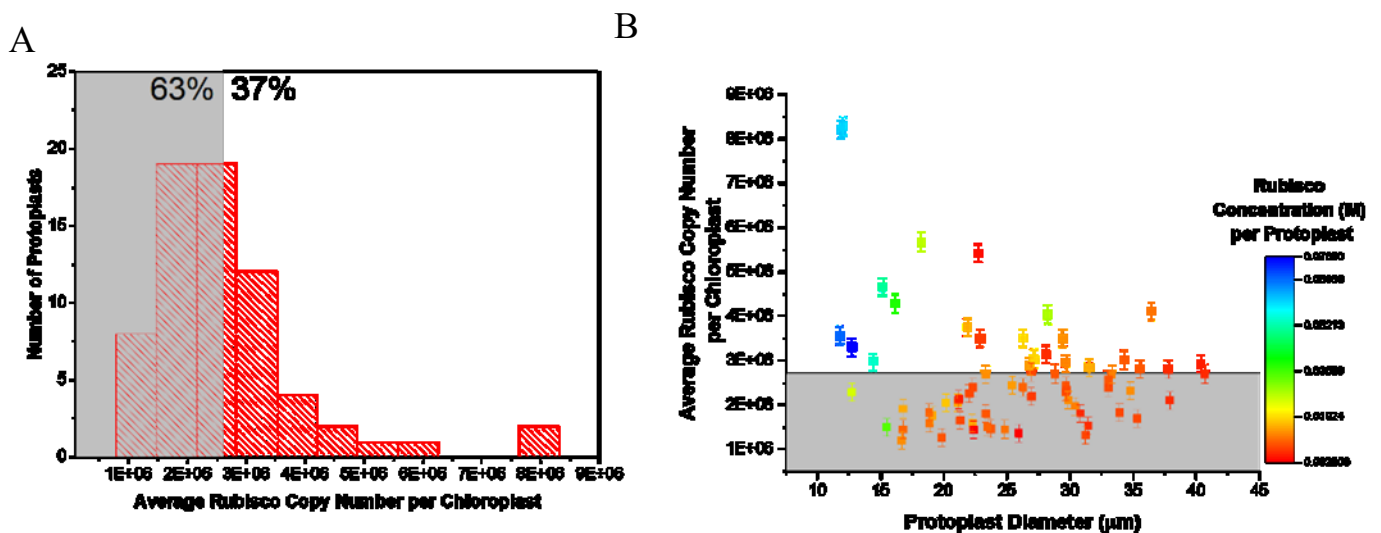


Figure 3.12 Calculated Rubisco Content per Chloroplast. By measuring the number of chloroplasts within one protoplast the average Rubisco content per chloroplast can be derived. From these 68 single chloroplast calculations a histogram of Rubisco expression (A) shows the average Rubisco content is 2.7 ± 0.2 million as denoted by the shaded region. 63% of the calculated chloroplast reside below this line and reside within protoplasts that have reached the maintained Rubisco concentration of 8×10^{-3} mM (B).

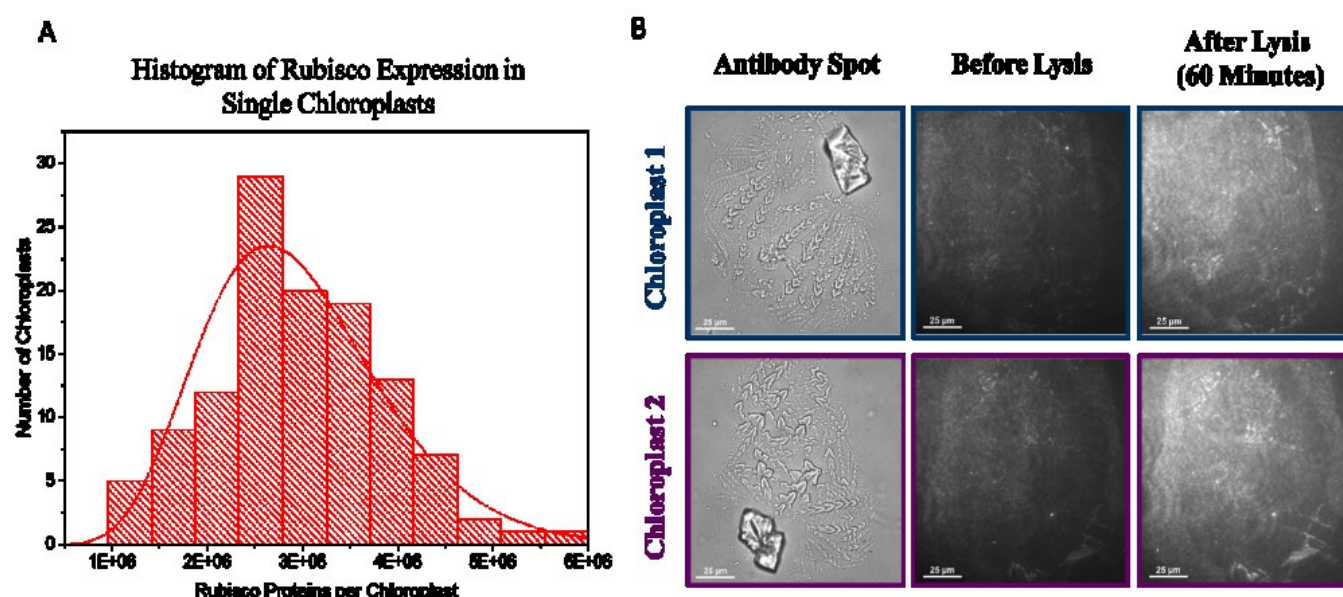


Figure 3.13 Histogram of Rubisco Expression. (A) The single organelle distribution of absolute Rubisco expression measured in 0.75 nL MAC chips, fit to a Gamma probability distribution. Within spinach leaves of different developmental stages chloroplasts express a range of Rubisco from 1 to 6 million Rubisco molecules, with a gamma distribution shape parameter of 11.94. (B) Raw image data from two chloroplasts are shown, that is from left to right a 60x brightfield image of the Array-it printed capture antibody spot. Followed by a before chloroplast lysis image and 60 minutes after lysis 60x Total Internal Reflection Microscopy image (scale bar is 25µm). The increase in fluorescence is recognized as single molecule Rubisco binding events.

3.5 Single Chloroplast Rubisco Measurements

It became apparent that to truly understand the mechanisms that were driving Rubisco expression and the noise associated, the organelle that was responsible for the final step of Rubisco assembly would need further analysis [155]. It has previously been postulated that the quantitative analysis of protein expression noise in an organelle distribution is a powerful way to probe the plight of single cell heterogeneity. As organelles, such as mitochondria can present an extrinsic source of cellular noise that contributes to the overall protein expression noise from a distribution of single cells [170]. Chloroplasts have shown, by analytical bulk measurement techniques or widefield fluorescence measurements, to alter the products of gene expression quantitatively and qualitatively during leaf development [171,172]. These gene alterations may have consequences that resonate from organelle to single cell.

Through implementing the calibrated Rubisco assay within 0.75 nL chambers, we have measured the Rubisco expression from single organelles. This Rubisco expression was additionally interrogated by comparison of organelle volume, calculated from deconvolved z-stack images. To further this ascertained information the organelle volume was treated as a continuous dependent variable and was associated with two independent variables the cpDNA concentration and Rubisco concentration of a chloroplast, allowing the evaluation of possible correlations within this and other published experiments [173].

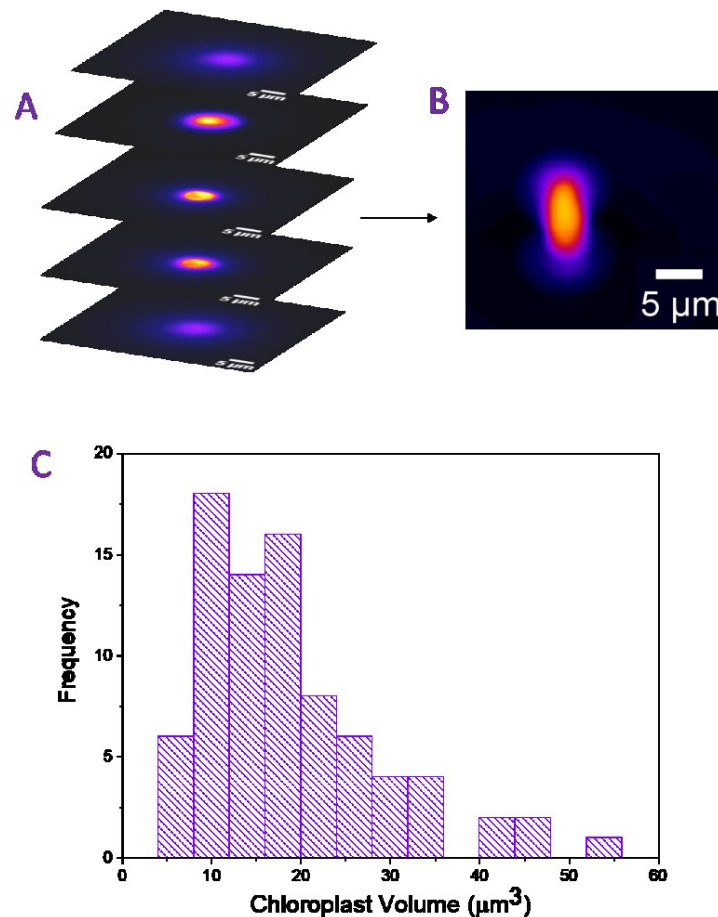


Figure 3.14 Volume Measurements of Chloroplasts. To accurately measure the volume of a chloroplast (A) z-stack images of chloroplast autofluorescence were acquired. (B) Upon deconvolution of these z-stack images a 3D model of the chloroplast shape was reconstructed. Allowing measurements of total chloroplast volume as (C) displayed in a histogram.

3.5.1 Chloroplast Rubisco Content

A mixture of chloroplasts, irrespective of protoplast, was isolated following the protocol outlined in section 2.6.4. These leaves were grown under identical MLI conditions to the previous 77 mesophyll cells analysed. The chloroplasts were interrogated for Rubisco content, and the distribution can be seen in *Figure 3.13*. Chloroplasts displayed a range of Rubisco expression from 1 to 6 million with an average expression of 3.18 ± 0.1 million. This measurement is lower than our mathematical approach of 7 million, based upon Rubisco constituting 50% of total chloroplast protein. Higher than the bulk estimate of 2.7 million from the average chloroplast content in the same mesophyll cell.

This distribution, when described by the gamma PDF, contains an unusually short tail, described by $K=11.94$, in comparison towards the protoplast Rubisco distribution of $K=3.54$. We hypothesise that the shortened length of this tail may be an artefact created by Rubisco content partitioning during organelle division partnered with slower Rubisco synthesis rates after division. This is based on the already provided evidence in section 3.4.2 that increased

degradation can shorten the rubisco tail length, such that $K = 19.02$. Additionally, larger protoplasts contain more chloroplasts, due to chloroplast fission, and exhibit a decreased Rubisco concentration.

3.5.2 Chloroplast Volume Optical Measurements

To evaluate the concentration of Rubisco within a $\sim 5 \mu\text{m}$ single chloroplast highly accurate volume measurements were necessary, due to their cupped or lens-shape [140]. We utilised $0.3 \mu\text{m}$ z-stack measurements of chlorophyll autofluorescence (stimulated by excitation of 678 nm) generated from single chloroplasts. These measurements provide an accurate representation, comparative to previously published images of the overall chloroplast spheroid volume following the application of image deconvolution as shown in *Figure 3.14*, [81].

3.5.3 Chloroplast Volume vs Rubisco Copy Number

Through performing chloroplast volume measurements before the lysis and subsequent Rubisco analysis, we measured an average volume of $18.9 \pm 1.5 \mu\text{m}^3$, agreeing with previous average chloroplast volume measurement of $20 \mu\text{m}^3$ in *Zea mays* [174]. Applying these volume measurements to the Rubisco copy number measurements, we have determined the average *Spinacia oleracea* chloroplast contain $3.14 \pm 0.105 \text{ pg}$ of Rubisco, providing agreement with Rachel Leech's previous bulk rocket immunoelectrophoresis measurements of $2.64 \pm 0.533 \text{ pg}$ per chloroplasts in young *Triticum aestivum* [173]. Leech, in 1996, derived this number by running five separate rocket immunoelectrophoresis experiments with a total of ten leaves of a known cell age ($n=50$ leaves). As wheat tissue develops in a linear fashion one can convert the distance from the leaf base into cell age. As Leech understood the number of chloroplasts per cell, as this has a direct association with leaf age, the measured amount of Rubisco could be first divided by the average number of cells in the sample, then divided by the average number of chloroplasts per cell.

Furthering on from this experiment Leech performed immunolocalization on wheat leaf sections for Rubisco content in chloroplasts with the largest cross-sectional area per cell ($n=30$), for eight different cell ages as shown in *Figure 3.15A*. Through using chloroplast volume and the normalised measurement average, a visual comparison can be assessed between Leech's immunolocalisation data and data acquired by the MAC chip as shown in

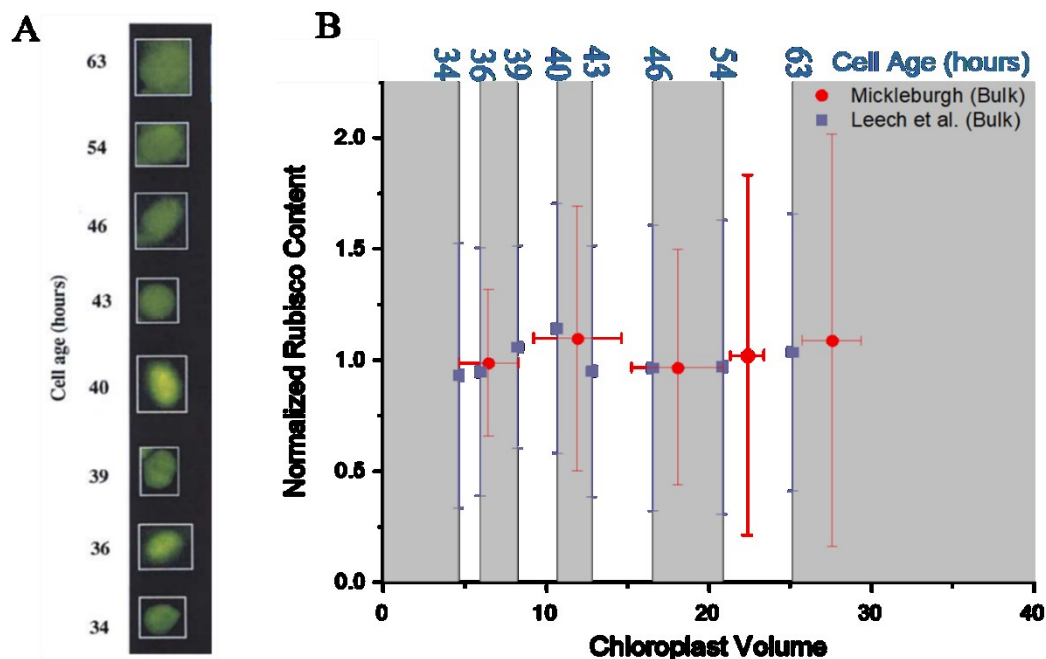


Figure 3.15 Bulk Chloroplast Rubisco Content Comparison. Leech et al. noticed a decrease in Rubisco content between bulk measurements of leaves assigned as 40 and 43 hours old. (A) Immunofluorescent localization in fixed tissue shows the Rubisco content for single chloroplasts presented a similar time associated expression pattern reproduced from (173). (B) By comparing average chloroplast volume between normalized Rubisco measurements from our data • and Leech et al. □ a similarity is shown in the drop in Rubisco content after 40 hours, explained by chloroplast division.

Figure 3.15 B. As Leech performed immunolocalisation experiments upon young developing tissue while our experiments were upon mature and developing tissue, only identical chloroplast volumes are compared. (i.e. Leech’s younger smaller chloroplasts have been omitted and our older larger chloroplasts have been omitted). Both bulk (*average*) data sets exhibit a striking similarity presenting that through chloroplast development, identified by an increase in volume, the Rubisco content decreases once. Leech explains this decrease as a consequence of chloroplast division, identified within cells of ~41.5 hours old.

As outlined in section 1.1, bulk (*average*) measurements upon a population of cells miss valuable information. By performing single-cell analysis, we gain the previously lost information of variability around this average measurement. As the chloroplasts analysed had two measured variables, chloroplast development and photosynthetic protein production. The application of a bivariate measurement technique provided a higher degree of data dimensionality (1-D to 2-D) which was assessed by a Gaussian mixture model (GMM) to assess the developmental aspects for photosynthetic protein production, essentially volume vs Rubisco content.

A GMM is a probabilistic model that groups data points based upon the number of specified components (k-value) residing in the entire data set. The k-value is user determined

and implies the number of Gaussian distributed distinct centroids (the average position), covariance matrices, and component proportions that reside within the data set while the value of L is the likelihood function for a model. To determine if the correct k -value has been input the use of the Akaike information criterion (AIC) can estimate the level of quality assurance between the GMM's, determined by *Equation 3.3*. AIC estimates the quality of a model to predict or estimate future values; a high-quality model is the one with a minimum AIC among all other models. Through fitting a GMM with k -values of 1, 2 and 3 to the overlapping data points of chloroplast volume ($4.5\text{-}34\text{ }\mu\text{m}^3$) vs Rubisco copy number we demonstrate as shown in *Figure 3.16* that the k -value 2 provides the best quality GMM for the overlapping data points, as the AIC value is lower, with respect to the alternative k -values 1 and 3.

$$AIC = 2 * \ln L + 2 * k \quad (3.3)$$

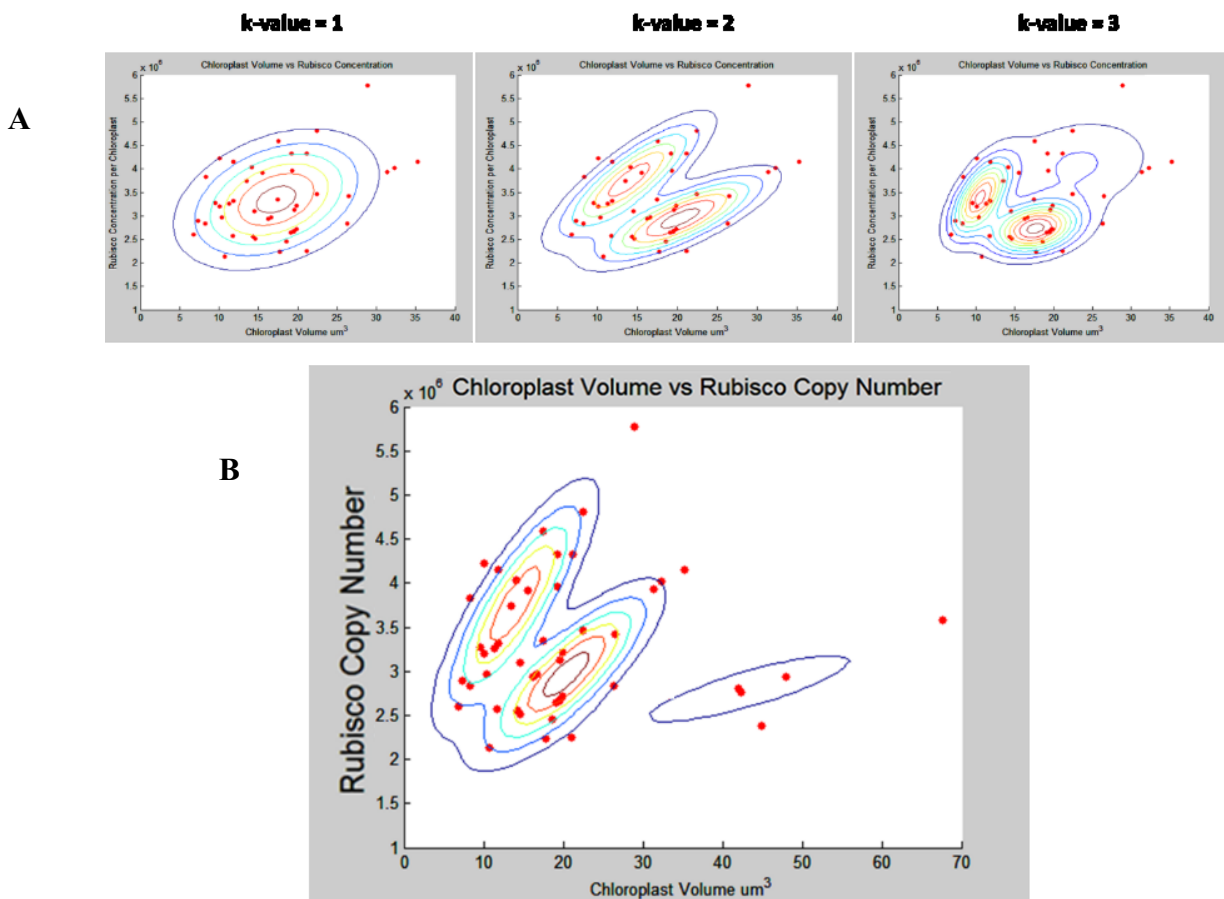


Figure 3.16 Gaussian Mixture Model Fitting. Through modifying model components, the k -value, (A) upon the Rubisco copy number data sets for chloroplasts with volumes from $4.5\text{-}34\text{ }\mu\text{m}^3$ the k -value 2 presented the lowest AIC criterion, providing evidence of two populations. (B) Upon the integration of the third population, arising from chloroplasts greater than $34\text{ }\mu\text{m}^3$, a k -value of 3 provides identical demarcation of chloroplasts smaller than $34\text{ }\mu\text{m}^3$ and identification of the third population previously evident by visual characterization.

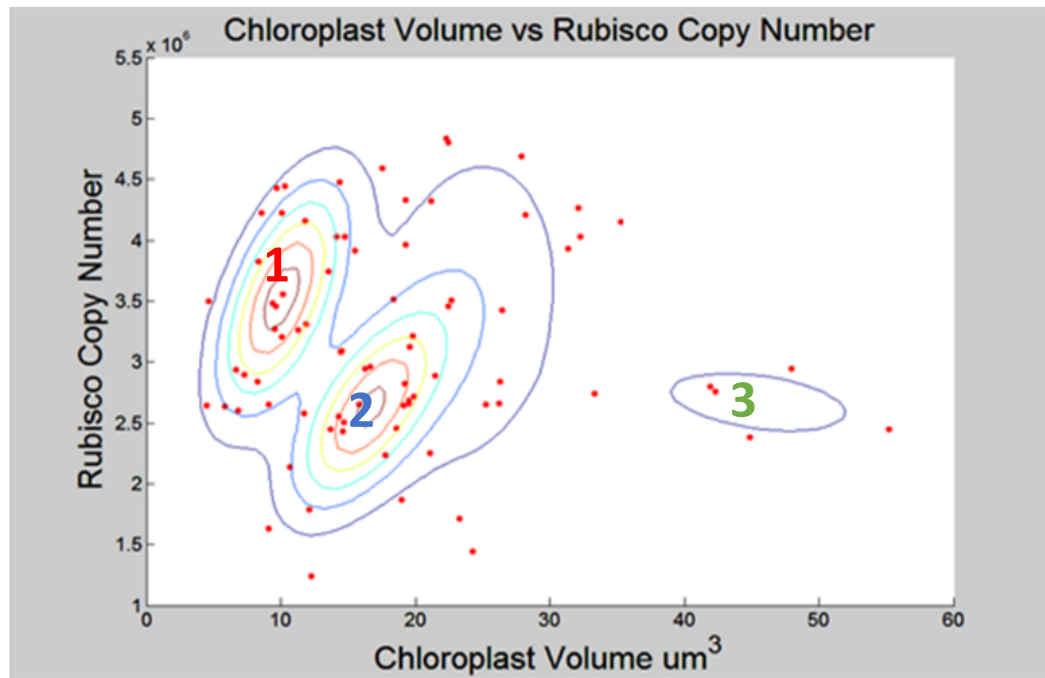


Figure 3.17 Robust Gaussian Mixture Model K-value 3. Through increasing the number of single chloroplast measurements to 81, the robustness of the model can be assessed. As the k-value 3 still provides the lowest AIC, while upholding similar identity values in comparison to the previous model selection, the assurance quality of the fit for three populations is high. The three populations are identified numerically nearby the population centroid.

We chose to limit the volume to $4.5\text{--}38\ \mu\text{m}^3$ as these volumes were the values previously compared with Leech's data. When we introduce the data for volumes $< 38\ \mu\text{m}^3$ and specify a k-value of 3 the GMM identifies the same two populations with a third population that was previously visually acknowledged as a sub-population. To increase the robustness of the model another MAC Chip experiment was performed ($n=35$) leading to a total of ($n=81$) data points that fit within the same three identified subpopulations, as shown in *Figure 3.17*.

3.5.4 Why Three Subpopulations?

To identify the cause of these three populations. Previous publications were reviewed, and three theories were derived for further investigation.

(1) The initial theory was the isolation procedure was producing intact chloroplasts (containing stromal material and an outer membrane) while also producing disrupted chloroplasts (no outer membrane and loss of stromal material) essentially just thylakoid membranes [138]. In 1968 Rachel Leech identified two damaged populations of wheat chloroplasts created due to isolation methods, one population exists between 0 and $11.2\ \mu\text{m}^3$ and the other between 35 and $55\ \mu\text{m}^3$. The first population existed as only thylakoid membranes and made a minimal contribution to Leech's size distribution pattern, while the latter exists as chloroplasts which maintain stroma content but are devoid of the outer

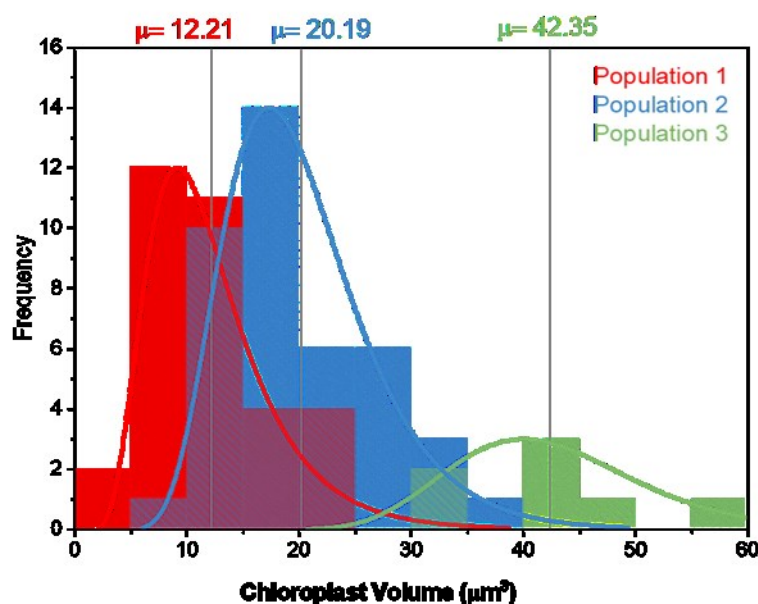


Figure 3.18 Chloroplast Volume Respective of Identified Populations The three populations identified by the GMM show different average volumes of 12, 20, and 42 μm^3 corresponding of population 1, 2 and 3. The average volume of population 3 shows similar characteristics to chloroplasts devoid of an outer membrane as previously characterized by the literature.

membrane [175]. In 1969 Karlstam et al. analysed spinach chloroplasts for integrity after isolation by counter-current distribution and characterised three populations, one agreeing with Leech, swollen and devoid of the outer membrane. The other two populations were identified as intact chloroplasts and given the title class IA and class IB. The class IB showed higher prominence in cotyledon measurements compared to developed leaves. Furthermore, it was postulated that the protein composition of the outer membranes of class IA compared to IB chloroplasts is different [176]. Leading us to dismiss the third population identified, as shown in *Figure 3.18*, as it was apparent, these cells were devoid of the outer membrane, and as discussed in section 2.6.4, our isolation technique would cause $\sim 12\%$ chloroplasts to be devoid of an outer membrane.

(2) The second theory was that the two populations were created due to a mixture of sun and shade exposed spinach leaves. The chloroplasts found within sun leaves contain poorly stacked grana and higher photosynthetic competency, displayed by an increase in Rubisco content. In shade leaf chloroplasts contain large and well-developed grana with a lower Rubisco content and lower photosynthetic output [177]. However, as the Spinach implemented in these experiments is grown under a directionally controlled light source and each plant is spatially separated, this may not appear to be a suitable explanation for the subpopulations in our data.

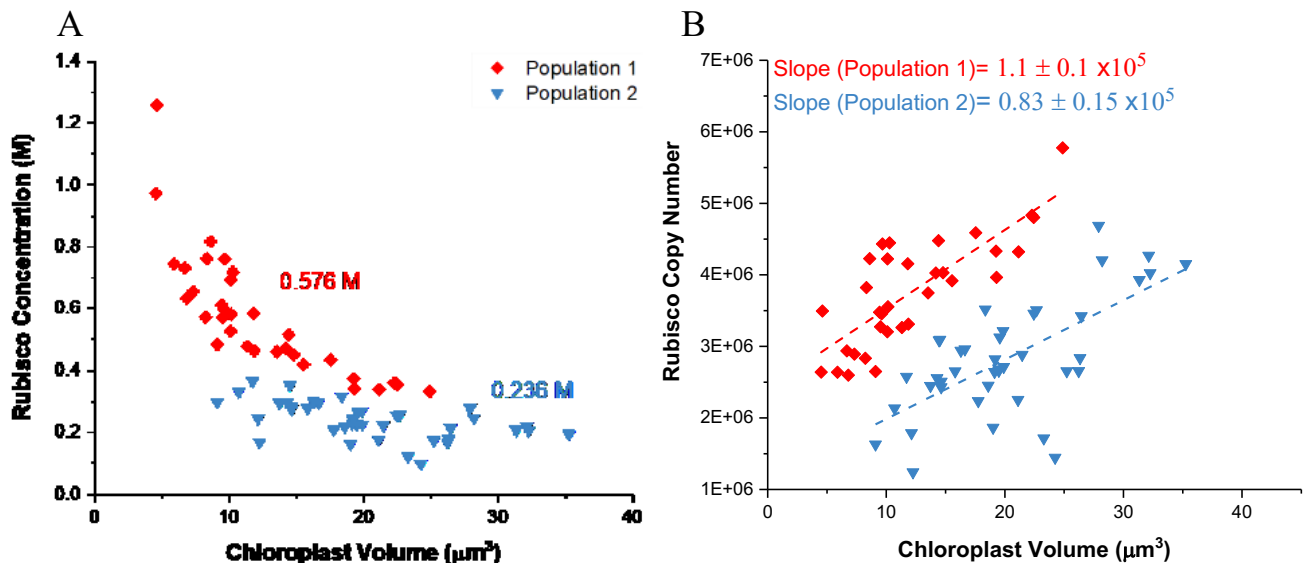


Figure 3.19 Rubisco Concentration for Population 1 and 2. Populations of chloroplasts identified by the GMM show altered average Rubisco concentrations. (A) Population 1 chloroplasts contain 0.58 M of Rubisco while population 2 contains approximately half with 0.24 M of Rubisco. (B) While the rate of Rubisco expression, estimated by the slope of each population decreases from population 1 to population 2.

It was previously identified that sun and shade chloroplasts could be found within one spinach leaf, where the sun chloroplasts were designated to reside in the palisade mesophyll cells, and the shade chloroplasts were within the spongy mesophyll cells [178]. The reported Rubisco concentration ratio between the spongy mesophyll chloroplasts compared to the palisade mesophyll chloroplasts was 0.48, this shows a high similarity to the data shown in *Figure 3.19A* where the ratio between population 2 and population 1 is 0.46.

(3) The third theory to explain the two populations is the process of chloroplast development from young to mature chloroplasts. Rachel Leech previously identified a slight decrease in bulk chloroplast Rubisco content between 37 to 41 hours-old leaf tissues through rocket immunoelectrophoresis, previously presented in *Figure 3.15*. This decline is best represented as a 50% Rubisco reduction in the post-divisional chloroplast. Upon chloroplast division, two daughter chloroplasts of equal volume emerge due to the highly regulated division machinery that forms around the FtsZ ring [179,180]. Comparison of the mean values between the two populations shows that population 2 is double the volume and contains half the Rubisco concentration comparative to population 1.

3.5.5 Chloroplast cpDNA Content Compared with Rubisco Content

To further understand if these populations arise through chloroplast development or are specific to cell type, a “marker” for chloroplast development would need to be identified and quantified. Chloroplast DNA (cpDNA) content appeared to be an appropriate “marker” as previous research by John Victor Possingham in 1980 provided evidence that Spinach cpDNA

content between young and fully developed leaves remained constant while the number of chloroplasts increased [181,182]. Implying that the number of plastomes per chloroplast decreased as protoplast aged. Further evidence of young chloroplasts in a protoplast containing all the necessary cpDNA for future created chloroplasts was provided through DAPI-DNA staining, and real time-quantitative PCR on immature and mature Arabidopsis leaves allowing cpDNA analysis [172].

Through implementing Hoechst, a DNA intercalating fluorescent dye, the marking of cpDNA from chloroplasts isolated using the previous technique described in section 2.6.4, could be assessed. In a similar manner to John Victor Possingham's experiments on spinach chloroplasts and Rachel Leech's experiments on wheat chloroplasts [181]. As shown in *Figure 3.20 B*, the total fluorescent intensity (A.U) per chloroplast was divided by the total chloroplast volume (μm^3) to calculate the measured cpDNA concentration (A.U) per chloroplast. Upon chloroplast volume comparison of the compiled measurements from cpDNA concentration and the previously measured Rubisco concentration, a high correlation ($R^2 = 0.95$) is witnessed, see *Figure 3.21*.

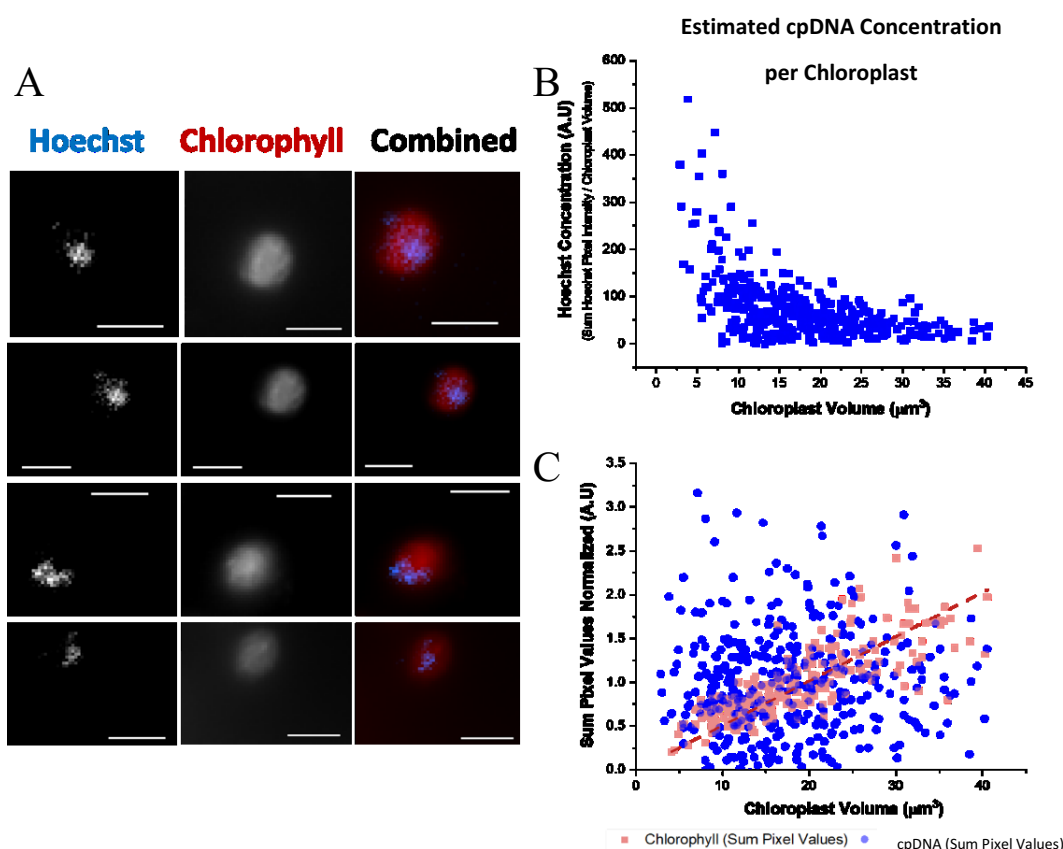


Figure 3.20 cpDNA Concentration Measurements. Through performing epi-fluorescent microscopy measurements upon a population of chloroplasts residing upon a coverslip the total cpDNA content and chlorophyll content per chloroplast can be estimated. (A) 60x epi-fluorescent image of the Hoechst intercalating fluorescent dye (excitation at 358 nm), followed by chlorophyll autofluorescence (excited by 650 nm) allows an estimated cpDNA concentration measurement (B). Concentration is defined as the summated Hoechst pixel intensity divided by the chloroplast oblate spheroid volume measured from the radius of chlorophyll area.

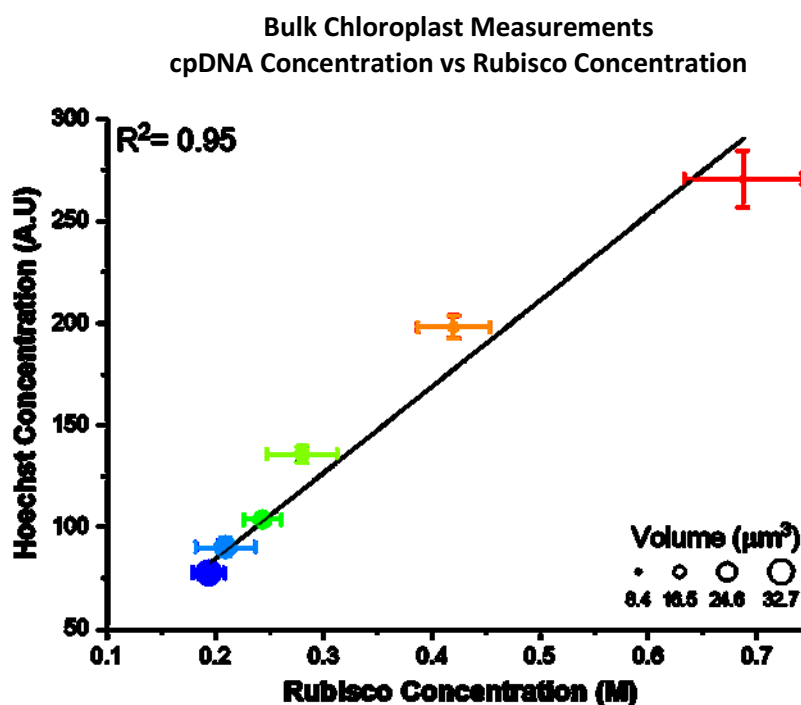


Figure 3.21 Average cpDNA Concentration vs Average Rubisco Concentration. The bulk average Rubisco concentration correlated with the bulk average cpDNA concentration by the constant experimental variable of chloroplast volume ($R^2=0.95$).

However, by measuring cpDNA content as a “marker” for chloroplast development with the expression of Rubisco neither theory 2 or 3 can be proven valid. In theory 2, it could be possible that the chloroplasts found in spongy mesophyll cells (population 2), consistently contain less cpDNA through development in comparison to the palisade mesophyll chloroplasts (population 1). Therefore, to provide evidentiary support for the correct theory, it is necessary to analyse the Rubisco content per chloroplast within a single protoplast, allowing us to identify if both or one chloroplast population is present.

3.6 Single Cell Multiple Organelle Rubisco Measurements

As new tools emerge that can be incorporated into microfluidic environments, the opportunity to probe different aspects of the biological landscape emerge. One of these tools, optical tweezers, has already provided a new biophysical characterisation of the interaction between chloroplasts and peroxisomes or cytoskeleton [140,141]. As the use of optical tweezers within the MAC chip is commonplace for the isolation of single cells or as previously shown single chloroplasts, it was apparent that this tool could be implemented to measure single chloroplasts from a single cell to provide insight into the subcellular behaviour of chloroplast-level Rubisco processes.

3.6.1 Operational Procedure

The implementation of the Rubisco quantification assay was unchanged; however, the trapping of the protoplasts, protoplast lysis, and subsequent chloroplast manipulation was altered, such that ~3 protoplasts were individually chosen from a population of protoplasts residing in the main chamber. These individually chosen protoplasts were optically trapped, as discussed in section 2.7.1, and moved into individual MAC Chip assay chambers as shown in *Figure 3.22A*. The main chamber was flushed (4% BSA/W5 with 1×10^7 molecules/nL Fitzgerald detection antibody) until no evidence of the previous protoplasts or cell debris was present. Next, protoplasts were optically manipulated from the MAC Chip assay chambers into the connection with the main chamber, seen in *Figure 3.22B*. Optical lysis was performed such that the cavitation bubble was produced 10 μm behind the protoplast to release chloroplasts into a defined area within the main chamber, presented in *Figure 3.22C*. This manner of chloroplast release was performed due to problematic previous experiments, where protoplasts were lysed within the MAC Chip assay chambers causing the inability for further chloroplast optical manipulation. This can be explained by chloroplast interactions upon the coverslip surface or that the release of the protoplast cytosolic contents reduced the refractive index mismatch with the chloroplast which facilitates optical trapping.

Individual chloroplasts were selected, based upon accessibility, such that chloroplast stromules can be disassociated allowing only single chloroplasts to be interrogated for Rubisco content. On average 35% of the total chloroplasts from a single protoplast were analysed for volume by this operational procedure and followed by Rubisco quantification through the miniaturised ELISA assay.

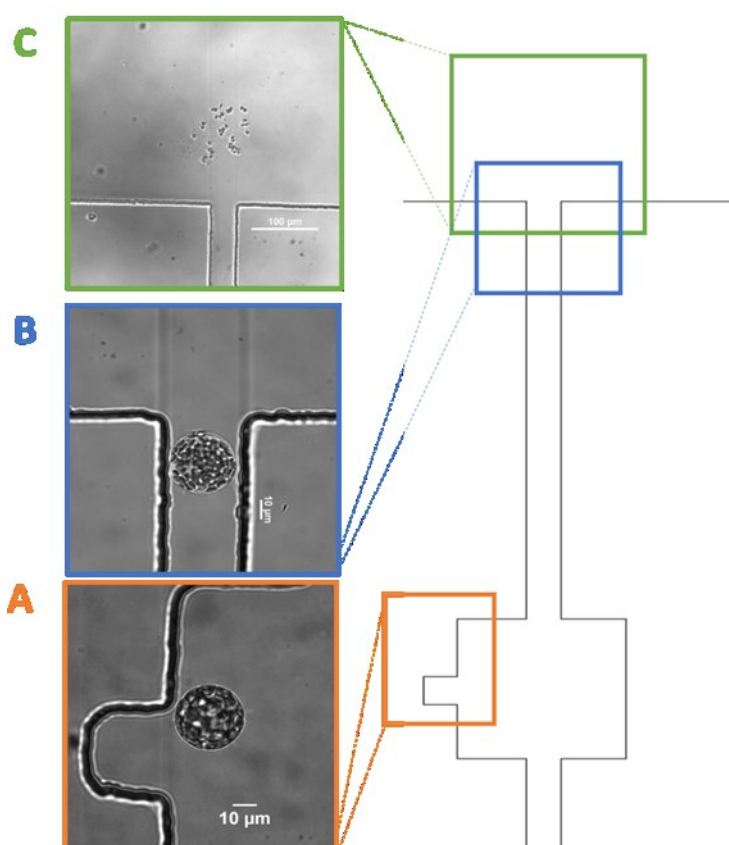


Figure 3.22 Graphical Depiction of Operational Procedure. Protoplast were optically trapped and segregated into MAC Chip Assay chambers (A). The main chamber is flushed, and the protoplast is optically manipulated towards the intersection (B). Optical lysis ensues, liberating chloroplasts into a known area of the main chamber (C) for further single chloroplast Rubisco assay interrogation.

3.6.2 95 Chloroplasts from 11 Protoplasts

A total of 11 protoplasts were analysed, with an average of 35% of the total chloroplast population being analysed. This led to a total of 95 chloroplasts being measured for Rubisco content, as shown in *Figure 3.23*. These chloroplasts share a similar distribution to the previously analysed chloroplasts which were irrespective of protoplast as previously shown in *Figure 3.13*. The mean Rubisco content of the 95 chloroplasts is 3.12 ± 0.1 million Rubisco per chloroplast.

Interestingly, 4 of the 95 chloroplasts expressed either above or below the previously measured range of Rubisco expression from 1 to 6 million, thereby extending the measured range to 0.5 - 6.8 million. It is evident by the total 211 chloroplasts measured that Rubisco expression in a single chloroplast is under a highly controlled mechanism of expression, represented by the variation in the gamma distribution is minimal (i.e. shape parameter). This variance-to-mean relation of expression shows a great similarity to that of the expression of ribosomes, another multi-subunit protein [183]. Furthermore, this shape parameter is recognised as a universal trend characteristic of other highly expressed proteins or highly regulated protein expression in bacteria and yeast [184,185].

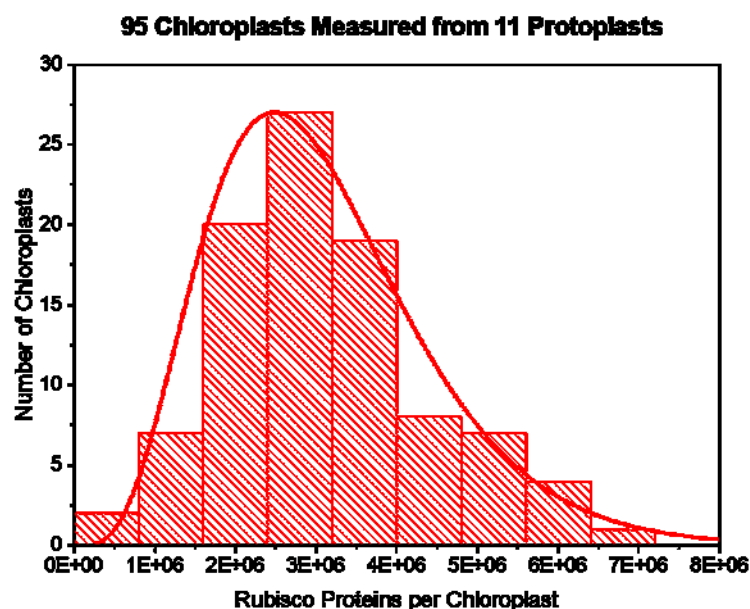


Figure 3.23 Histogram of Rubisco Expression from 95 chloroplasts isolated from 11 Protoplasts. The distribution of chloroplasts analyzed from the 11 protoplasts show an identical, to previously measured chloroplasts, average Rubisco copy number of 3.12 ± 0.1 million and fit to identical gamma probability distribution shape and scale parameters. It is apparent after sampling 211 chloroplasts that the short tail in Rubisco expression is not an artifact of experimental under-sampling.

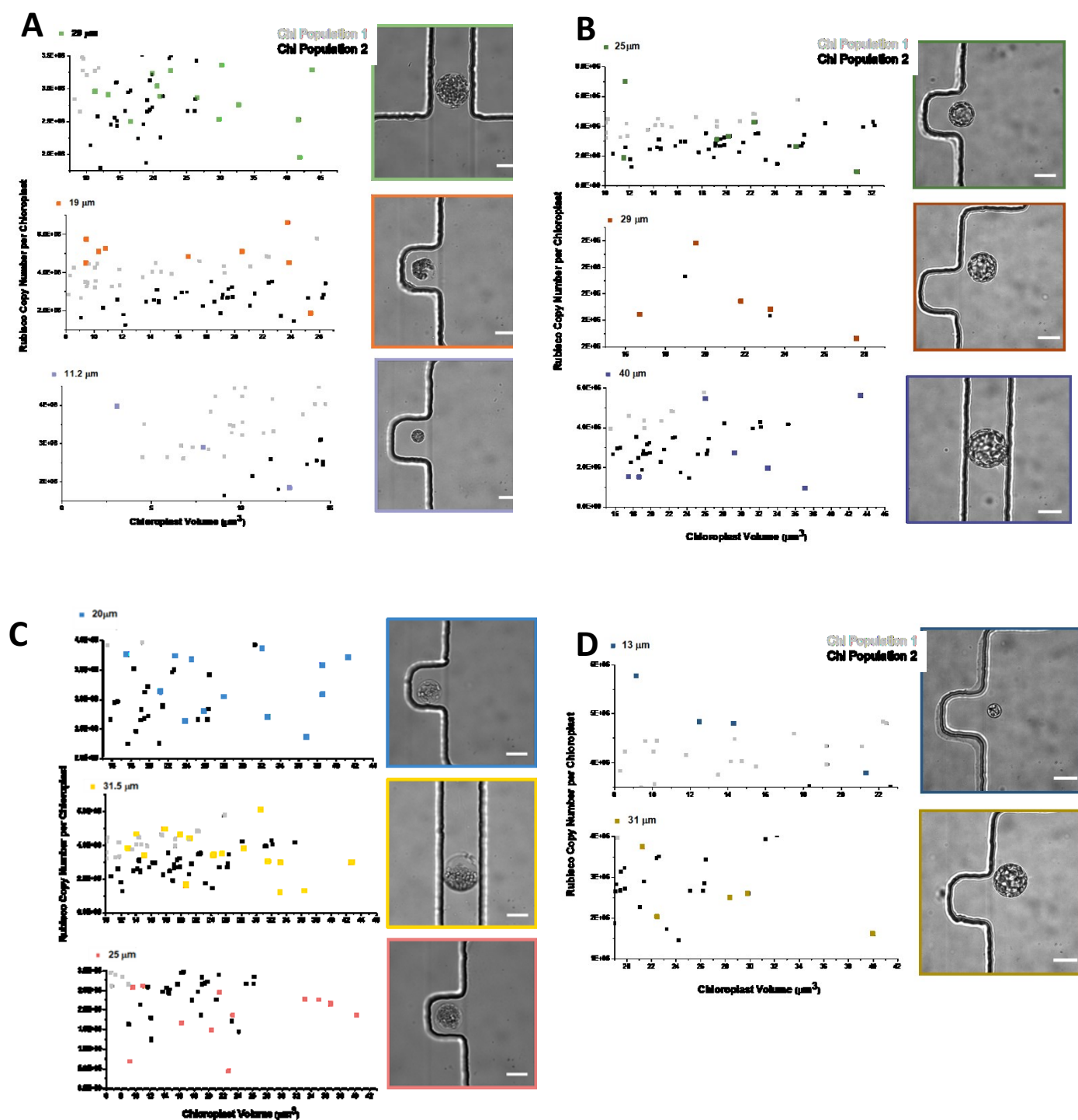


Figure 3.24 Single Cell Multiple Organelle Rubisco Quantification. Four 0.75nL MAC Chip experiments were performed notated as A, B, C and D. A range of 11 protoplasts were sampled from 11-40 μm in diameter, each protoplast is pictured in a 60X brightfield image (scale bar 20 μm). For locational referencing the chloroplast population 1 and 2 are displayed yet not graphically scaled towards. Scaling is with respect to the chloroplasts measured for volume and Rubisco content from the single protoplast.

In 81% of the protoplasts examined chloroplasts could be identified to exist in both GMM identified populations, as shown in *Figure 3.24*. However, from the average sample of 35% of chloroplasts present within the protoplast, a clear majority can be identified as belonging to only one population. Upon assessment of protoplast size with respect to the chloroplast majority (defined by the average measurements of chloroplasts respective to protoplast), an association between protoplast size and the chloroplast population identity can be witnessed. This association is displayed in *Figure 3.25*. The smaller protoplasts containing smaller volume chloroplasts, hence younger in development, and contain a chloroplast majority which resides within chloroplast population 1 (The GMM previously defined this population in section 3.5.3). The larger protoplasts which contain larger volume chloroplasts, thus older in development, contain a chloroplast majority which resides within chloroplast population 2 (The GMM previously defined this population in section 3.5.3) .

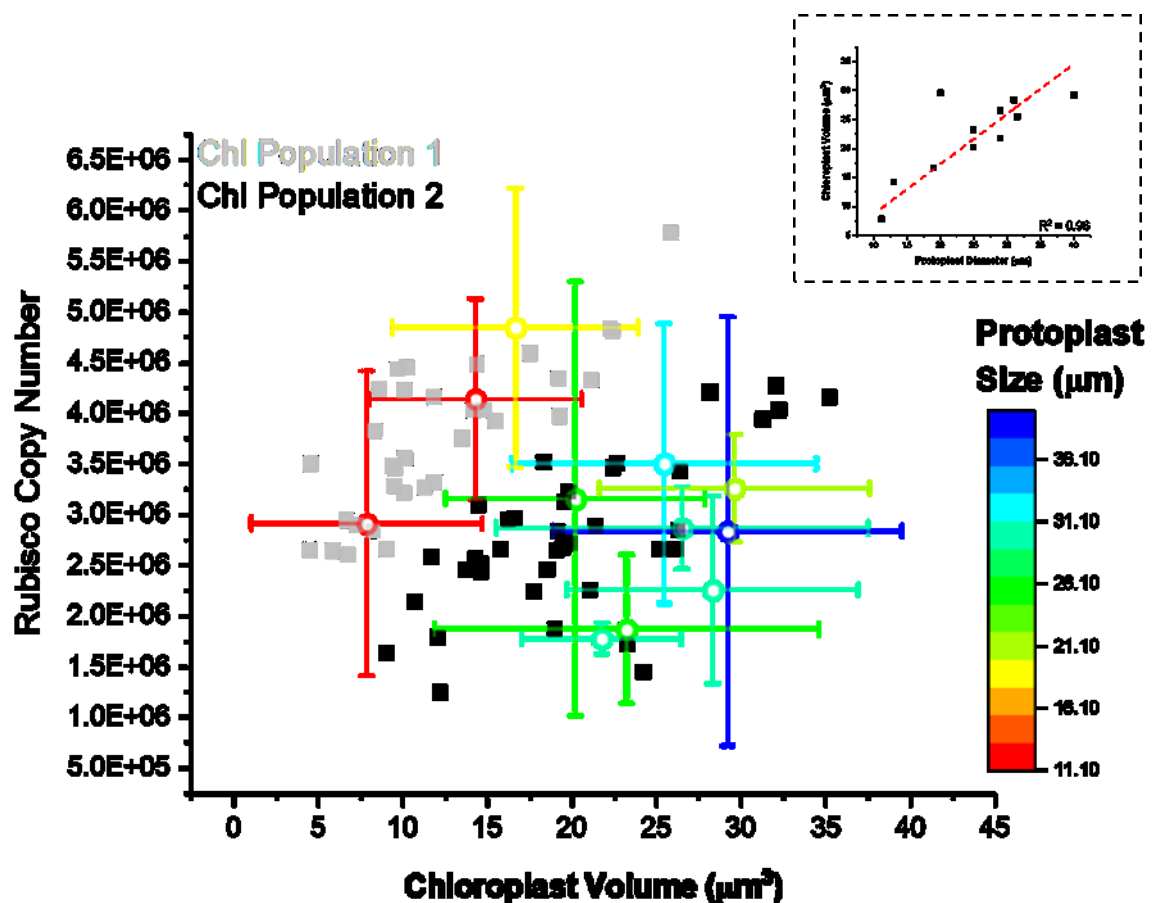


Figure 3.25 Chloroplast Average Behavior with Respect to Protoplast Size. After plotting the average chloroplast volume and Rubisco copy number respective of the protoplast origination. A high linear correlation ($R^2 = 0.96$) between protoplast size and average chloroplast volume, presented in the insert, is measured. This is descriptive of protoplast and chloroplast maturation. Furthermore, through maturation the chloroplast majority can be seen to move from the designated population 1 to population 2, these populations were previously designated by the GMM, discussed in section 3.5.3.

3.6.3 A Chloroplast Conclusion: One Chloroplast Is Not the Other!

The ability to measure multiple chloroplasts from a protoplast has allowed us to provide evidence that each cell can contain chloroplasts which reside in both populations. However, as a chloroplast majority is predominantly found within one population, this does not provide sufficient evidence to discern between theory 2 or 3. Yet, the chloroplast majorities existence within a population is associated with cell size. As both palisade and spongy mesophyll cells undergo size associated development a range of different cell sizes for both cell types would be present, therefore if theory 2 were correct the cell size association with chloroplast majority population designation would not be witnessed.

It appears theory 3, the process of chloroplast development, can be reasoned by previous literature and our experimental evidence to describe the production of two Rubisco content distinct subpopulations of chloroplasts. In 1969 by Karlstam et al., the two identified spinach chloroplast populations class IA and class IB measured by counter-current distribution displayed different protein composition within the intact outer-membrane. This differing protein composition could be rationalised by slower protein production rates after chloroplast division, as we have demonstrated in Rubisco production. Rachel Leech, in 1996, measured a divisional reduction in Rubisco production, however, due to bulk measurements the creation of the biochemically altered subpopulation was buried within the average measurement. Similar to what we have measured in *Figure 3.10 B*, due to a lack of single organelle resolution only an increase in Rubisco was measured throughout protoplast development.

Advancing towards single organelle resolution, we provide evidence that *one chloroplast is not the other!* We have identified that outliers do exist towards the choice of existence in the two biochemical subpopulations. These outliers provide support for theory 3, such that if Rubisco expression were dependent upon development, outliers from the chloroplast majority would exist due to the asynchronous developmental aspects of chloroplast division [147]. Furthermore, upon the exclusion of theory 2 further evidence towards the creation of subpopulations by development is presented by a high association of the declining concentration of Rubisco with the declining concentration of cpDNA. In conclusion, these two chloroplast populations presented in *Figure 3.26* exhibit altered Rubisco content and Rubisco expression noise. This increase in Rubisco noise (σ^2/μ^2) from population 1 (0.04) to the after division population 2 (0.07) shows a resemblance to that of a decreasing mRNA content [77].

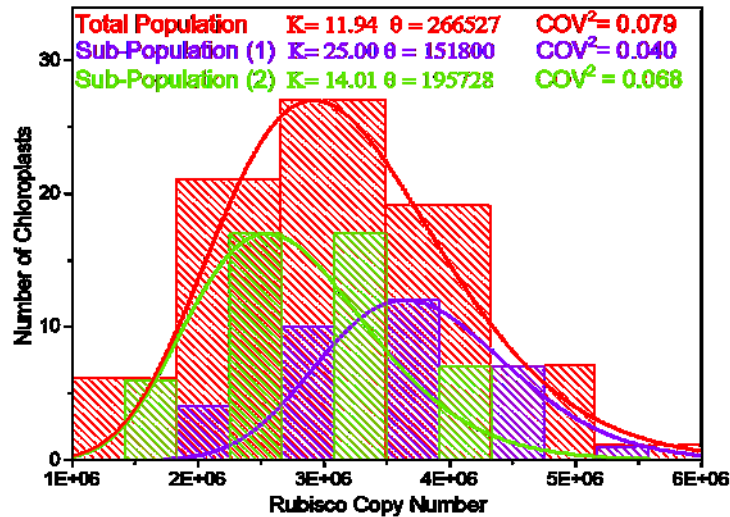


Figure 3.26 Two Chloroplast Populations Defined by Altered Rubisco Expression. The two identified populations originating from an event of chloroplast division show an altered Rubisco expression noise, fit to a gamma distribution PDF and defined by COV^2 . Such that population 1 is 0.04 and population 2 is 0.07. This increase of noise is reminiscent of low copy number protein expression investigated by Vahid et al, whereby an increase in mRNA content results in a decrease of protein expression noise (42).

Furthermore, we see that population 1 maintains 0.11 ± 0.01 million Rubisco molecules per $1\mu m^3$ grown while after division population 2 sustains 0.083 ± 0.01 million Rubisco molecules per $1\mu m^3$ grown as previously shown in *Figure 3.19B*. These chloroplastic measurements highlight the existence of biochemically dissimilar chloroplasts which appear to have gone by the wayside through previous bulk chloroplast measurements. Opening a new path of investigation towards answering, why does a protoplast contain heterogenous chloroplasts, who controls this deviation within the cell and if/how regulation is imposed.

3.7 Measurements of Rubisco with Rubisco Activase

Rubisco is predisposed to the production of catalytically stalled complexes. Such that RuBP binds to the uncarbamyated Rubisco or CA1P binds to the carbamyated Rubisco at night [186]. To overcome these catalytic inefficiencies, Rubisco maintains a relationship with a partner protein RCA. RCA is an AAA+ chloroplastic protein, it implements an ATP-dependent reaction, resulting in unfolding the C-terminal domain of the Rubisco large subunit, thereby releasing the inhibitory sugar complex trapped within Rubisco [187]. RCA comprises approximately 2% of the soluble protein in spinach leaves, thereby from Equation 3.1 and 3.2 we estimate an abundance of 3.5 million RCA molecules per chloroplast [188]. To further probe this partnership, we expanded upon the original Rubisco assay to include measurements of bound RCA. This allowed investigations towards the association between Rubisco and RCA concerning stages of leaf senescence.

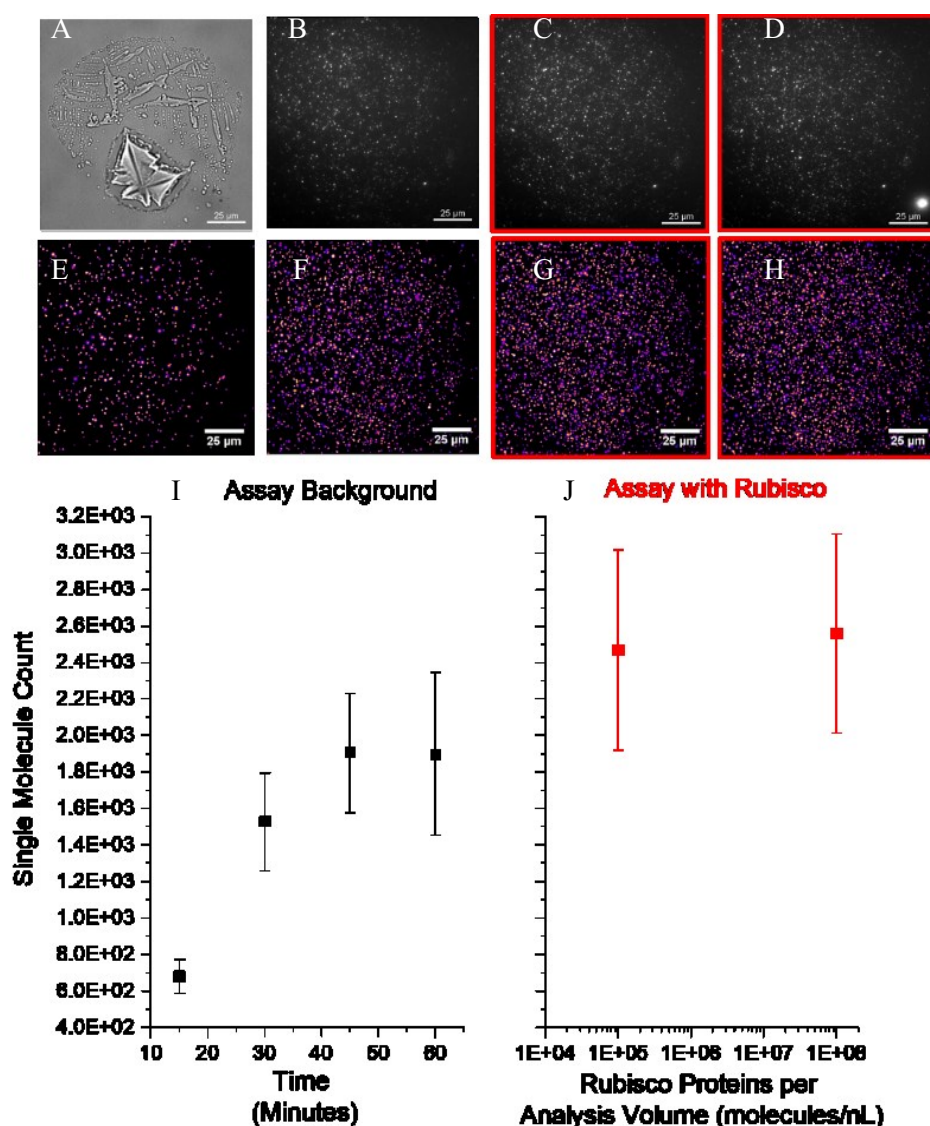


Figure 3.27 RCA Detection Antibody Preliminary Characterization. Implementing open chip experiments to assess the NSB between the fluorescently labeled anti- RCA antibody and the anti-Rubisco capture antibody. (A) 60x Brightfield image of the printed Array-it capture antibody. (B) Followed by raw acquired images for the same spot at 60 minutes NSB and 60 minutes with 1×10^5 and 1×10^8 Rubisco per nL (C, D). (E, F) The single molecules identified by peak fitting for 15 minutes and 60 minutes NSB, (G, H) followed by 60 minutes with 1×10^5 and 1×10^8 Rubisco per nL. The NSB between the two antibodies reaches equilibrium before 60 minutes (I) and shows little increase of binding events upon the addition of Rubisco (J).

3.7.1 Preliminary RCA bound Measurements

By modifying the choice of secondary antibody, in the original Rubisco quantification assay, to an anti-RCA secondary antibody (Item No. sc-15864; Santa Cruz Biotechnology) we can measure the RCA bound to Rubisco. Preliminary experiments, shown in *Figure 3.27*, provide evidence of non-specific binding (NSB) between this new secondary antibody and the original Rubisco capture antibody, which reaches equilibrium well before 1 hour. Furthermore, these binding events do not significantly increase from the NSB upon the addition of 1×10^5 Rubisco molecules/nL and 1×10^8 Rubisco molecules/nL, providing evidence for lack of affinity of this RCA antibody towards Rubisco.

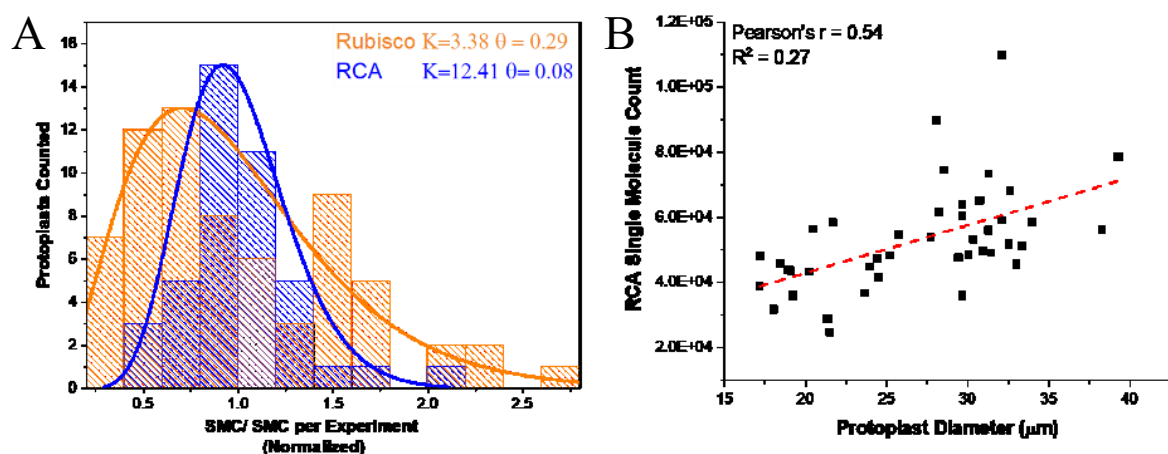


Figure 3.28 The 1st Experiment RCA Bound Single Protoplast Measurements. (A) By performing 0.75 nL MAC chip experiments with the modified Rubisco assay a smaller variance-to-mean relationship for single molecules counted (SMC) is shown for RCA bound ($K=12.41$) compared to the previous Rubisco measurements ($K=3.38$). (B) Also, RCA bound exhibited a decrease between the dependence of single molecules counted and protoplast diameter (Pearson's $r=0.54$) compared with Rubisco (Pearson's $r=0.74$).

The first experiment following the general operational procedure outlined in section 2.8 allowed the identification of RCA bound to Rubisco for 42 mesophyll protoplasts. These protoplasts isolated from Spinach following the isolation protocol described in section 2.6.2 presented a smaller distribution variance of RCA in comparison with the previous measurements for Rubisco content, *Figure 3.28 A*. Furthermore, an intriguing decrease in linear correlation between bound RCA and protoplast size was displayed (Pearson's $R=0.54$) *Figure 3.28 B*. To further understand this relationship between RCA and Rubisco content a multiplexed assay was designed.

3.7.2 Rubisco and Rubisco Activase Multiplexed Measurements

To further probe the distribution of RCA bound to Rubisco, multiplexed measurements were performed, defined as introducing both secondary antibodies into the assay, the Santa Cruz antibody for RCA and the Fitzgerald antibody for Rubisco, at concentrations of 1×10^7 molecules/nL. Western blot evaluation for the specificity of the two antibodies was performed by implementing the methods outlined in section 2.3.5, shown in *Figure 3.29*. Two bands can be identified, the previously identified band from Rubisco at ~ 55 kDa followed by a band below ~ 53 kDa, this band may be representative of the larger RCA isoform which has been shown to be ~ 45 kDa [187].

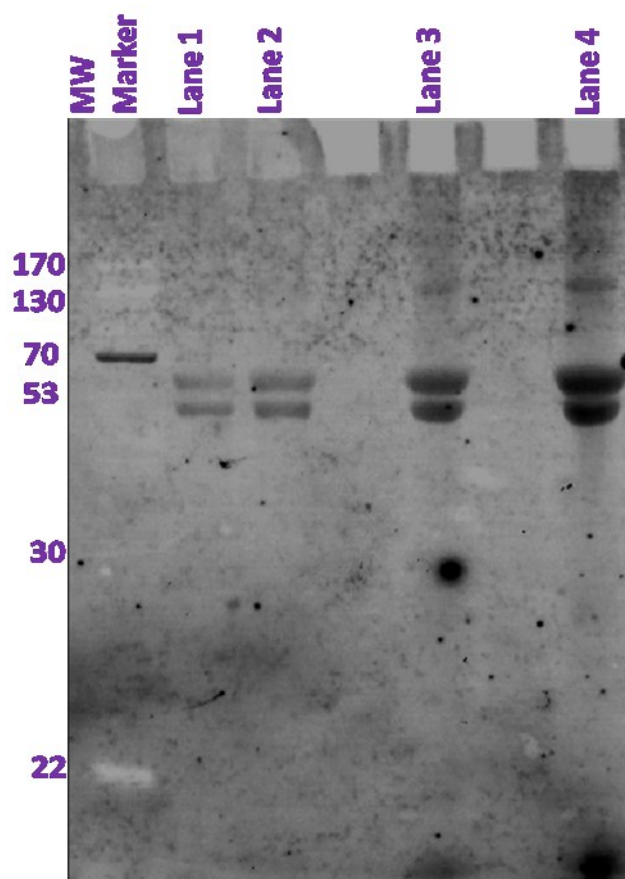


Figure 3.30 Multiplexed Lysate Western Blot. To assess the affinity of multiplexed assay measurements upon Rubisco and RCA, spinach cell lysate samples of increasing cells/mL (Lane 1-4) were probed with the Fitzgerald anti-Rubisco antibody and Santa Cruz anti-RCA antibody conjugated with FITC and AlexaFluor 488 respectively. Two prominent bands were identified at above 53 kDa and below 53 kDa, representative of large subunit Rubisco (55kDa) and large isoform of RCA (45kDa)

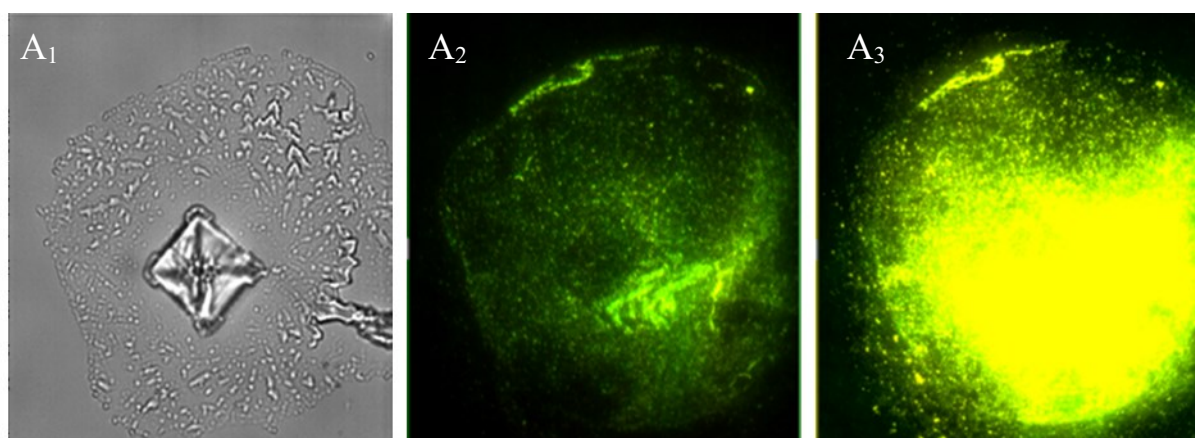


Figure 3.29 Multiplexed Rubisco and RCA Bound Assay. Raw experimental images for one protoplast assay are presented, such that green denotes the Rubisco FITC secondary antibody and orange denotes the RCA AlexaFluor 546 secondary antibody, images for Rubisco quantification and RCA bound quantification were acquired separately but have been merged for presentation. (A₁) 60x brightfield Array-it capture antibody spot. (A₂) Followed by a merged 60X TIRF illuminated image of secondary antibody (Non-specific Binding) equilibrium after 60 minutes and the (A₃) final merged image binding events of Rubisco and RCA bound after assay equilibrium.

Multiplexed measurements were performed on Spinach, that was grown under the conditions specified in section 2.6.1. This long day condition approach for illumination induces flowering on older plants and in-turn senescence in their older leaves. Allowing two populations of protoplast for analysis, those isolated from yellow senescing leaves and those from healthy green leaves producing a fixed plant developmental time point. From the 7 spinach plants analysed one of the plants is shown in *Figure 3.31 A*. As previously discussed, the dynamics of Rubisco synthesis and degradation should be dissimilar in the two populations.

Upon the analysis of the green photosynthetically active leaves, a correlation is witnessed ($R^2 = 0.70$; Pearson's $R=0.84$) between the measured content of Rubisco and amount of bound RCA, as presented in *Figure 3.31 B*. This bound RCA distribution shows similar gamma PDF parameters to the previously assessed preliminary RCA measurements.

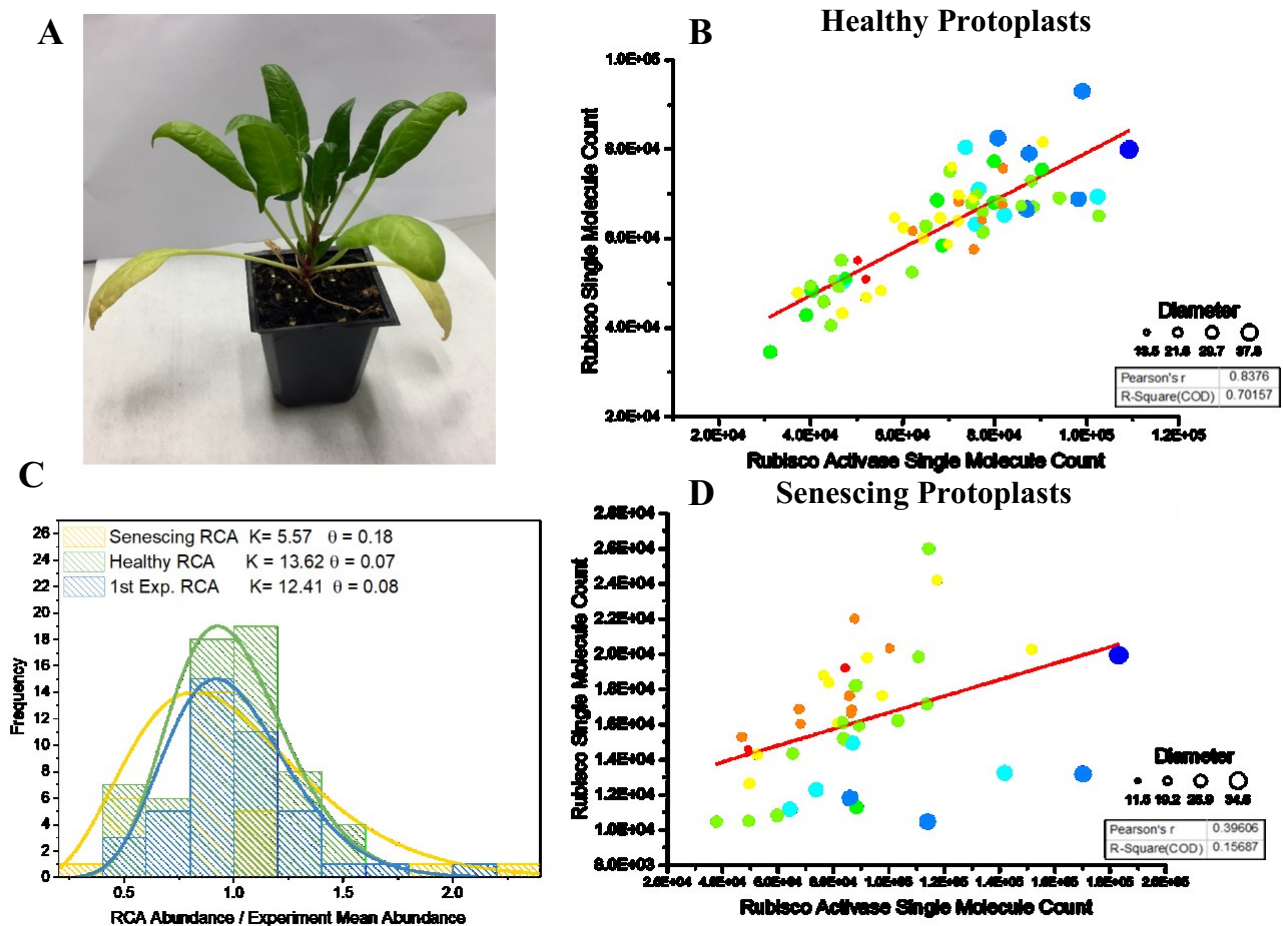


Figure 3.31 Multiplexed Measurements of Rubisco and RCA bound for Healthy and Senescent Protoplasts. Spinach grown under long-day conditions allowed separate experiments upon newly developing leaves near the stem apex and senescent leaves in the basal region of the stem (A). The healthy leaves exhibited a higher correlation between RCA bound and Rubisco ($R^2= 0.70$) (B) and produced a similar RCA bound distribution to that measured in the 1st RCA bound experiment, where Rubisco content was not measured (C). The senescent leaves showed very little correlation between Rubisco and RCA bound ($R^2= 0.15$) whereby RCA bound shows a greater variance to mean distribution ($K= 5.57$) and Rubisco content is higher in smaller protoplasts (D). Protoplast size is denoted by circle diameter and color.

Senescing yellow spinach leaves, which were undergoing Rubisco degradation, present a decrease in correlation ($R^2 = 0.16$; Pearson's $R = 0.40$) and an alteration in the bound RCA distribution presented in *Figure 3.31 C* and *Figure 3.31 D*. It was the increase and variation within the bound RCA distribution for senescing leaves which was rather perplexing, as the ratio of RCA to Rubisco measured on the capture antibody spot increased from 1.09 ± 0.02 in healthy leaves to 5.6 ± 0.33 in senescing leaves as shown in *Figure 3.32*.

This increase of RCA association with Rubisco for senescent protoplasts could be reasoned from previous microtiter sandwich ELISA experiments. Showing evidence that a lack of ATP causes an increased association between both proteins, significantly when Rubisco is uncarbamylated. Furthermore, coprecipitation experiments have shown evidence that the presence of ATP decreases the presences of coprecipitates with RCA [189,190]. This could be an indication that the ratio of ADP to ATP is altered from healthy to senescent protoplast. Another explanation for this increased association could be RCA displaying characteristic chaperone behaviour. RCA has shown a higher affinity for forming complexes with non-native Rubisco compared to native Rubisco [191]. To fully understand this association there is a need for further experiments; the ability to determine the absolute values of RCA and the addition of ATP into the MAC Chip assay chambers would need to be performed.

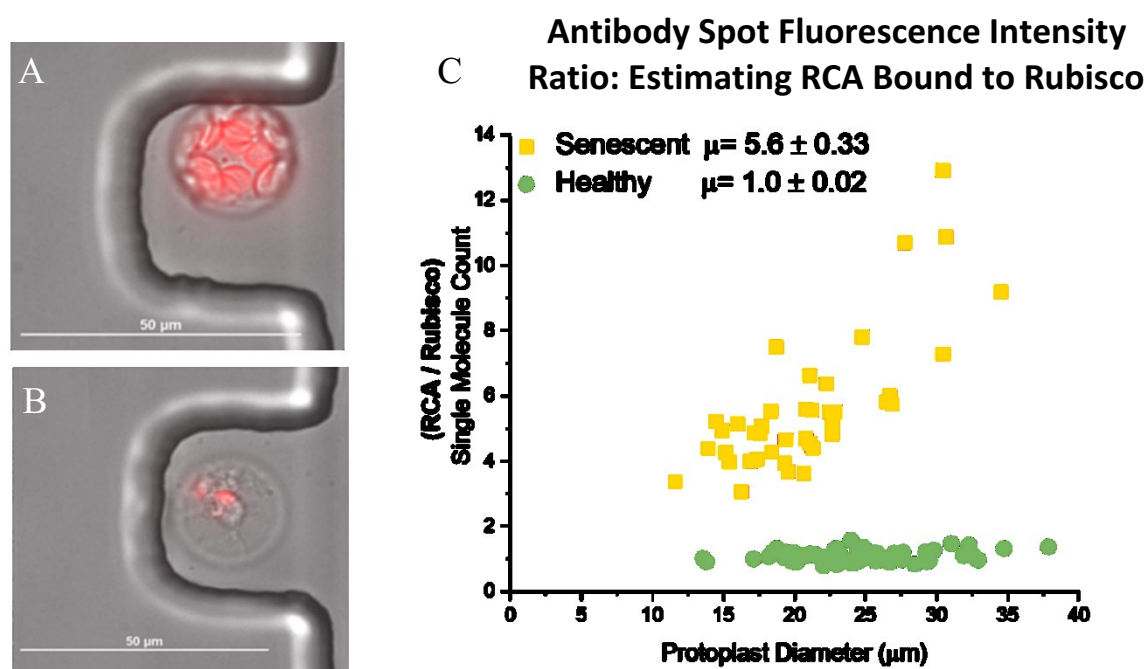


Figure 3.32 Arbitrary Fluorescence Ratio for Estimating RCA Binding Events to Rubisco. At single cell resolution the chlorophyll and chloroplast number can be seen to decrease from (A) healthy mesophyll cells to (B) senescent mesophyll cells. This decrease is accompanied by a decrease in Rubisco content, as during senescence Rubisco is degraded to salvage total leaf nitrogen content for newly developing leaves. (C) An increase in the antibody spot fluorescence ratio descriptive of a presence of RCA bound to Rubisco are seen for senescent cells (5.6 ± 0.33) compared to healthy cells (1.0 ± 0.02).

3.8 Conclusion

The development of a Rubisco single molecule quantification assay integrated into sub-nanoliter assay chambers has provided technological advancements allowing the developmental trajectories of Rubisco at the single cell and organelle resolution. This technology was first implemented to assess single mesophyll protoplast Rubisco distributions from spinach leaves under varying conditions, allowing an assessment of the sensitivity of the Rubisco quantification assay. The Rubisco distributions in *Figure 3.8*, representative of the HLI and MLI produced similar variance-to-mean relation (distribution shape) yet presented significantly different population means using Bonferroni test ($=0.05$) with average copy numbers $2.2 \pm 0.9 \times 10^7$ and $3.6 \pm 1.64 \times 10^7$ respectively. The Rubisco distribution for senescing protoplasts exhibited a reduction in variance-to-mean relation and an average copy number of $1.7 \pm 0.4 \times 10^6$, provided in *Figure 3.9*.

Further assessment towards leaf development and Rubisco expression provided insight towards a higher correlation between the number of chloroplasts present within a protoplast and the Rubisco copy number, rather than that of the protoplast diameter, as shown in *Figure 3.11*. To further understand the creation of these protoplast Rubisco distributions, measurements were performed upon the Rubisco expression from single chloroplasts. The ability to increase measurement dimensions by combining the measurement of chloroplast volume with Rubisco copy number, three populations could be identified through GMM fitting, presented in *Figure 3.17*. One population was discredited, as this resembled disrupted chloroplasts from previous literature. The other two populations were identified after a novel adaption of technique was implemented to analyse populations of chloroplasts isolated from a single protoplast. These single protoplasts multiple chloroplast measurements, shown in *Figure 3.25*, provided new insight towards the two populations showing a strong association with chloroplast development.

To gain further insight towards Rubisco expression through development, we performed multiplexed experiments, allowing the measurement of RCA bound to Rubisco. A correlation between Rubisco and RCA for photosynthetically active mesophyll cells was measured while senescent mesophyll cells presented very little correlation, see *Figure 3.31*. This lack of correlation presented a greater variance-to-mean relationship in the RCA bound distribution of senescent cells compared to the RCA bound distribution found in healthy cells.

Assessing single cell and organelle development in association with Rubisco copy number presented that the Rubisco content appears to be under a highly regulated mechanism represented by the variance-to-mean relationship displayed in Rubisco copy

number histograms ascertained from healthy protoplasts. Furthermore, the chloroplast distributional relationship of Rubisco expression shows a high regulation, similar to ribosomal expression, another multi-subunit protein [183]. Further evidence of this controlled expression is demonstrated in a decreased variance for Rubisco content, and the amount of RCA bound, based on the antibody spot intensity ratio, presented in *Figure 3.32*, there is 1.09 ± 0.02 RCA's present for each Rubisco. This developmental regulation of Rubisco appears to manifest as the protoplast "tuning" to a photosynthetically favourable Rubisco concentration of 7.69 ± 2 mM. The analysis of variability within the protoplast, hidden from previous bulk analysis methods, demonstrates this "tuning" is associated with a chloroplast division event. Identified as through protoplast development the chloroplast population transitions the Rubisco concentration from 0.576 M to 0.236 M, presented in *Figure 3.19*, thereby maintaining the catalytically optimal protoplast Rubisco concentration. However, in the end, senescence takes hold and degradation of chloroplasts and Rubisco dominates the Rubisco content measurements. This Rubisco degradation produces a variance-to-mean relationship representative of a decreased protein expression noise while based on the antibody spot intensity ratio there is an average 5.6 ± 0.33 RCA's bound to one Rubisco.

Chapter 4

Bulk Cell Analysis of 4-HPPD

This experimental chapter is focused on a key impediment towards the absolute proteomic applicability of the microfluidic affinity capture chip. This restriction is founded in the limited accessibility of antibody assays which can provide the requisite limit of detection for single cell protein copy number quantification. This concept is illustrated by the development of a tri-molecular antibody assay for 4-HPPD quantification, the assay integration for single cell analysis, and the transition towards bulk cell analysis after single cell detection limit preclusion. The bulk cell measurements in normal and etiolated *A. thaliana* mesophyll cells were found to be 271 ± 98 and 975 ± 100 4-HPPD molecules per cell, respectively.

4.1 Introduction

Hydroxyphenylpyruvate dioxygenase (4-HPPD) plays a crucial role in the biosynthesis of vitamin E (α -tocopherol) and plastoquinone. This crucial role arises due to the reaction product of 4-HPPD, homogentisate (HGA). HGA is the essential aromatic precursor for α -tocopherol and plastoquinone vital to the antioxidative system and photosynthetic electron transport chain respectively. 4-HPPD manufactures HGA through an oxidative decarboxylation step, where two oxygen molecules are incorporated by intramolecular hydroxylation, forcing a rearrangement upon the pyruvate side-chain presented in Figure 4.1 [192].

Motivation towards research upon this enzyme has been stimulated by two factors. The importance of vitamin E towards both human and plant physiology. In human physiology, a significant source of dietary vitamin E is acquired from plant foods. Elevated levels of dietary vitamin E results in decreased risk of certain cancers, cardiovascular disease and age-related decline of the immune system [193]. In plant physiology, 4-HPPD has become a crucial herbicide target. As inhibiting this enzyme leads to lethal photobleaching and a collapse of the photosynthetic electron transport chain due to the absence of plastoquinone (white leaves) [194,195]. However, this crucial target is becoming less viable due to a spread in herbicide resistance, such that *Amaranthus tuberculatus* within the United States has become resistant to Mesotrione (2- [4-(methyl sulfonyl)-2-nitrobenzoyl]- 1,3-

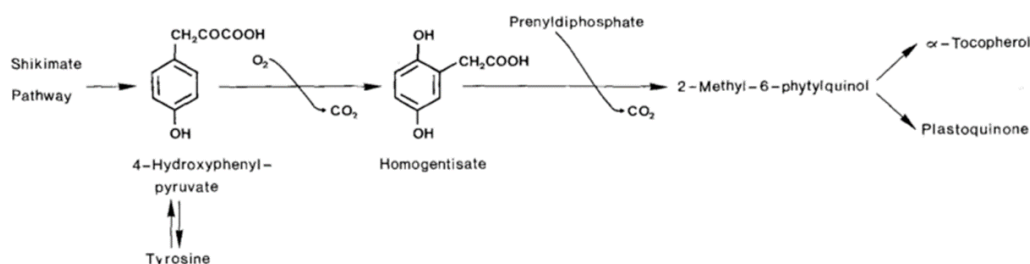


Figure 4.1 The Creation of Homogentisate. The role of 4-hydroxyphenylpyruvate producing homogentisate for the biosynthesis of vitamin E (α -tocopherol) and plastoquinone. Adapted from [193].

cyclohexanedione). Mesotrione is a member of the triketone class of 4-HPPD inhibiting herbicides [196]. While studies elucidating the possible mechanism of this resistance either by target site mutagenesis or possible detoxification mechanisms through enhanced metabolism, it is the latter that is rather alarming. Resistance conferred by an increased rate of metabolism can confer plants with forms of multiple herbicide resistance, which are far more genetically complex. Unfortunately, this is the case in McLean County, IL [197].

Through previous research into understanding the resistance towards the herbicidal method of action or research on healthy plants, three important molecular characteristics of 4-HPPD have been identified. The localisation, structure, and abundance. However, these measurements on 4-HPPD abundance have not been quantified in absolute terms. Debates have arisen concerning the subcellular compartmentalisation of 4-HPPD, as research has shown 4-HPPD can be found in the cytosol, chloroplasts, and peroxisomes. Furthermore, this subcellular localisation appears to be species specific such that in *Spinach oleracea* 4-HPPD is localised within the chloroplasts while in *Arabidopsis thaliana* 4-HPPD is only found within the cytosol, determined by fractionation followed by SDS-PAGE and immunocytochemistry on tissue sections [198,199].

Insight towards 4-HPPD structural information was previously estimated from the distantly related tetrameric enzyme from the chemo-organotrophic gram-negative prokaryotes. These estimates presented 4-HPPD as a homotetramer. This was followed by biochemical characterisation of the enzyme produced within eukaryotes, revealing the behaviour of a homodimer. In 2004 Fritze et al. presented crystal structures of *Arabidopsis thaliana* 4-HPPD at 3.0 Angstrom resolution. This presented evidence for the first time in plant biology that 4-HPPD was also a homodimer encompassing the active site composed of a nonheme ferrous iron centre. Providing insight towards access to this active site, such that access is restricted within *Zea mays* (a C-4 plant) while the active site is fully accessible in *Arabidopsis thaliana* (a C-3 plant) [199].

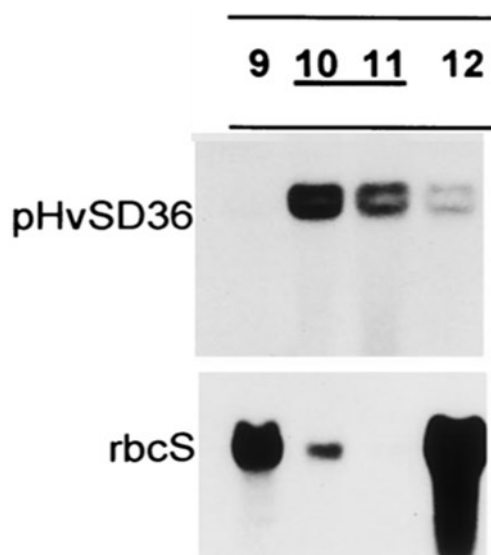


Figure 4.2 Comparative Northern blot analyses of pHvSD36 (4-HPPD) and rbcS (Rubisco small subunit) transcript levels. Northern blot analysis of 25µg of total RNA from primary foliage leaves from before (9) during (10,11) and after (12) the dark treatment. Adapted from [200].

The last discovered characteristic is the alteration of 4-HPPD expression concerning stressors upon leaf development. It has been well characterized that the final stage of leaf development, senescence, is a degradative process where new and specific gene expression is undertaken. 4-HPPD is one of these genes which undergoes alteration in genetic expression, such that elevated levels of mRNA, corresponding to sequence homology of 4-HPPD increase upon senescence, as reproduced in *Figure 4.2* [200].

Elevated levels of 4-HPPD have been ascribed essential towards the chloroplast degradation process. An increase in the antioxidant α -tocopherols allows a decrease in the photosynthetically derived free radicals (ROS), which upon chloroplast membrane degradation would induce lipid peroxidation if the ROS were allowed to scavenge freely [201]. The success in genetic expression measurements upon this alteration can be misinformed, such that senescence profiles induced by darkness (etiolation) rather than natural senescence (cell death) produce alterations in the genetic profile. Therefore, for clarity, the processes undertaken in this chapter involves etiolation rather than the approach towards Rubisco profiling in section 3.7 which involved natural senescence.

This chapter describes the creation of a 4-HPPD miniaturised detection assay and its implementation for single cell analysis. However, detection limitations precluded single cell analysis. Therefore, a transition towards bulk cell measurements was performed and provided first time quantitative insight towards the average bulk cell measurement content of 4-HPPD under normal and etiolated conditions for *Arabidopsis thaliana*. These measurements are found to be 271 ± 98 and 975 ± 100 4-HPPD molecules per normal and etiolated cell respectively.

4.2 Designing a 4-HPPD Assay

This section discusses the designing of a miniaturised assay for the detection of 4-HPPD. Achieved through the creation of a 4-HPPD recombinant protein coupled with a preliminary assessment of four anti-HPPD antibodies. Three of those antibodies, in various roles as either

primary or secondary antibody, presented viable candidates, yet only two of the combinations produced a linear increase in signal response with the increased addition of recombinant 4-HPPD. One of these combinations of antibodies, which detected 4-HPPD in a multimer condition, was improved to create the final 4-HPPD quantification assay. Improvements were created through manipulation of the capture antibody concentration.

4.2.1 Characterization of Recombinant Protein

Working with Dr Anthony Keeley, a member of Syngenta's protein science group at Jealott's Hill, recombinant 4-HPPD (His-AthHPPD-delta28) was manufactured. This was achieved by inserting a plasmid conferring antibiotic resistance and the relevant *Arabidopsis thaliana* genetic information for creation of His-AthHPPD-delta28 within *E. coli* BL21. A preliminary colony was grown upon agar plates, allowing only *E. coli* with bestowed antibiotic resistance to proliferate. This colony was transferred to culture and produced 60g of *E. coli* lysate.

The protein of interest, His-AthHPPD-delta28, was purified from this lysate by the implementation of an AKTA™ design FPLC system coupled with a His-Trap FF column (ThermoFisher, UK). The peak representative of His-AthHPPD-delta28 underwent centrifugal concentration, Vivaspins 500 (Sigma Aldrich, UK) and the protein concentration was determined by performing UV-Vis measurements in a nanodrop (ThermoFisher, UK) presenting a concentration of 24.36 mg/mL. To determine the purity of the sample 5 µL was run through SDS-PAGE following the practices outlined in section 2.3.5. After Coomassie Blue staining, a prominent band around 46.95 kDa representative of His-AthHPPD-delta28 is displayed. Presenting an 87.8% sample purity as displayed in *Figure 4.3*.

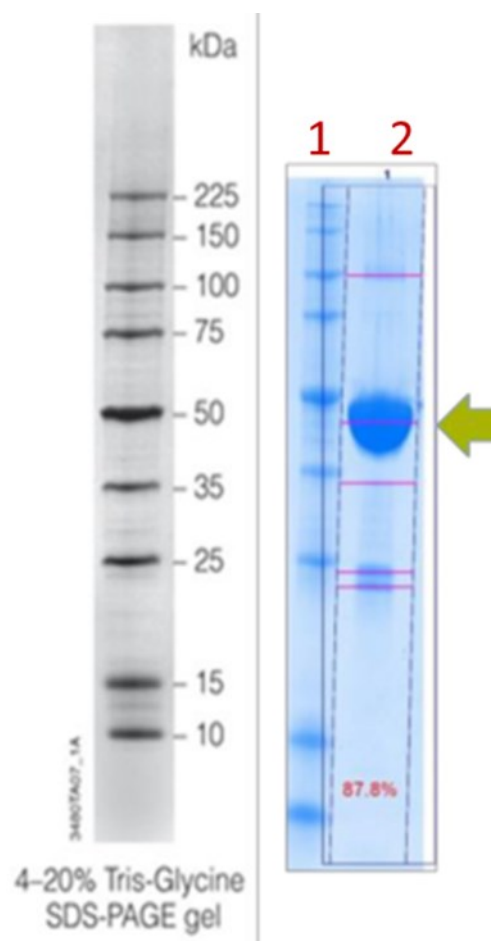


Figure 4.3 Purity Analysis of His-AthHPPD-delta28. The sample extracted from *E. coli* lysate was analyzed in lane 2 by polyacrylamide gel electrophoresis. Lane 1 is Promega broad range markers. The sample identified by Dr. Anthony Keeley is presented as a green arrow and is of 87.8% purity.

4.2.2 Preliminary Antibody Evaluation

A total of four antibodies, raised against a sequence of 4-HPPD from *Avena sativa*, were generously donated by Dr Michele Yarnall from Syngenta's

analysis group at Research Triangle Park. Two of the antibodies were monoclonal, designated as HPPD-15 and HPPD-19, while the last two were polyclonal antibodies designated as Rabbit Alpha and Goat Alpha. Syngenta's analysis group has measured the sensitivity of these antibodies in a plate-based ELISA assay. Such that coating with HPPD-15 and using HRP-HPPD19 for detection presents ~5 ng/mL sensitivity. While the sensitivity of coating with Goat Alpha and detecting with Rabbit Alpha present 1-2 ng/mL.

All antibodies were obtained as 2mg/mL in a volume of 0.5 mL PBS. Upon arrival half of each antibody stock was conjugated to AlexaFluor 488 (Item No. ab62391; Abcam, USA) by applying a conjugation reaction. The conjugation reaction consisted of the Lightning-Link® Rapid Conjugation System and was carried out as specified by manufacturer instructions (Innova Biosciences, UK).

Preliminary tri-molecular open chip assays were performed, where 30 μ L of 1×10^6 His-AtHPPD-delta28 (Δ) in a solution consisting of 4% BSA in W5 is pipetted upon a coverslip of microarray printed primary antibody (Y) printed at a final concentration of (0.5 mg mL^{-1}). Within this solution resides 1×10^7 secondary antibody (Y) conjugated to AlexaFluor 488, for protein binding event detection, as presented in *Figure 4.4 A*. These preliminary experiments, qualitatively assessed for yes or no binding after 60 minutes and presented three positive hits; HPPD15: HPPD19, Rabbit Alpha: HPPD19, and HPPD-19: HPPD-19. This last combination presents superficial evidence that the recombinant protein is present as a complex, as an identical monoclonal antibody was implemented for both capture and detection of 4-HPPD. Raw experimental images for each combination has been presented in *Figure 4.4 B¹B²B³*, respective of each antibody combination.

These three antibody combinations, which generated positive hits, were implemented for the production of a calibration curve. A method previously discussed in Section 3.3.6 for the protein Rubisco. MAC Chips, containing 0.75nL assay volumes were vacuum infiltrated with a solution of 4% BSA in W5 with the appropriate secondary detection antibody at a concentration of 1×10^7 . Each solution is spiked with His-AtHPPD-delta28 ranging from 1×10^3 - 1×10^8 molecules/nL. After 60 minutes the HPPD assay reaches equilibrium and is imaged for single molecule binding events. The resulting Raw, non-background subtracted calibration curves, consisting of the known number of HPPD molecules/nL and the number of single molecule binding events are presented in *Figure 4.4 C*. The first striking result, is that the positive hit measured in the open chip experiment for HPPD15: HPPD19 was not a measurement of protein binding, but that of secondary antibody interactions with the printed primary antibody. The Rabbit Alpha: HPPD-19 combination presented only one order of

magnitude detection capabilities, while HPPD-19: HPPD-19 presented around three orders of magnitude for 4-HPPD detection. A detection capability which is similar to the developed Rubisco Assay, p53 Assay, and GFP Assay.

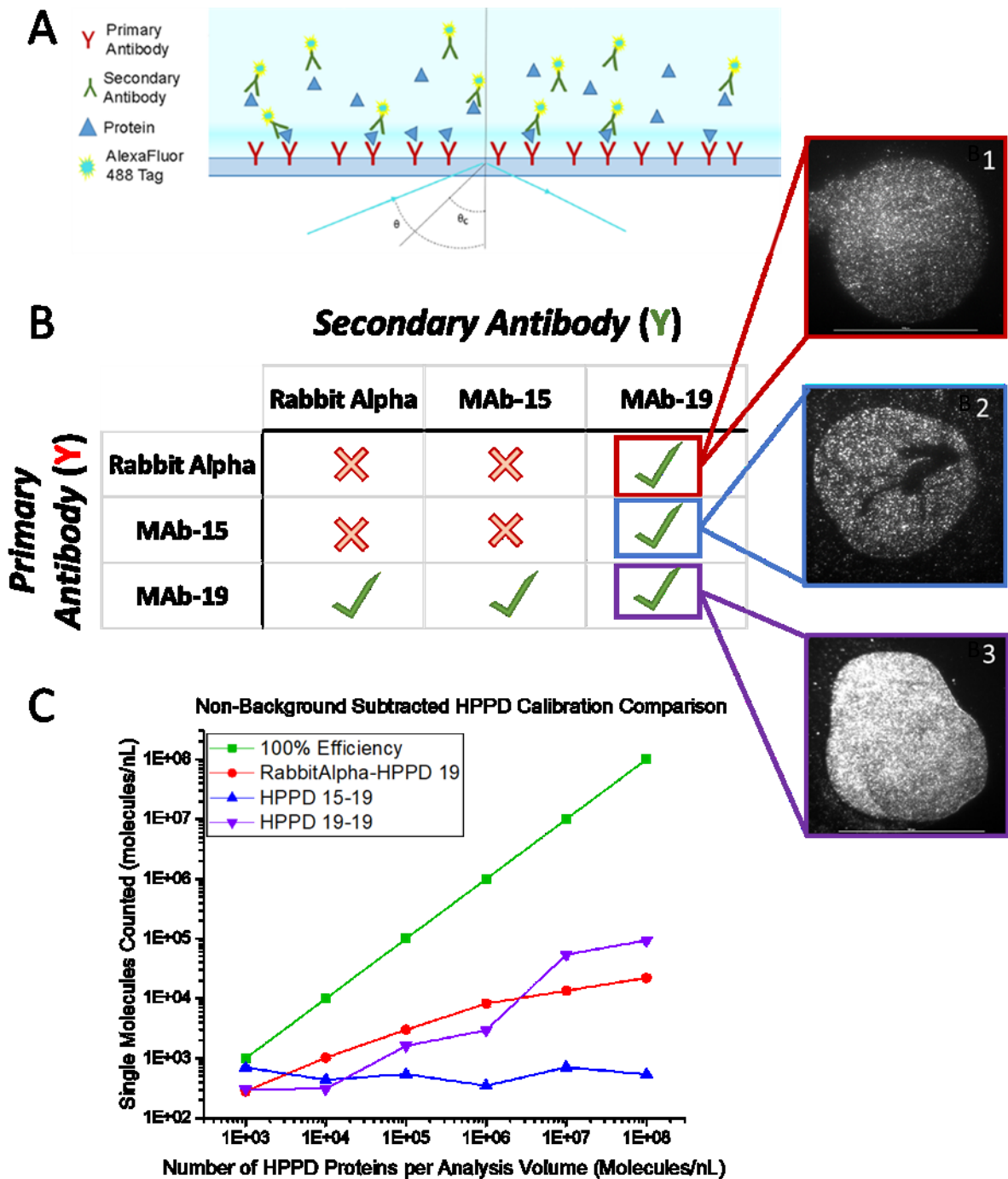


Figure 4.4 The selective screening process of multiple antibody combinations towards 4-HPPD. Development of a miniaturized assay for protein detection involves the selection of two antibodies with a high avidity towards the protein of interest. Antibodies can be either free in solution coupled with a fluorophore, the secondary antibody or are immobilized on the coverslip surface, the primary antibody (A). Upon open chip interrogation with the presence of 1×10^6 4-HPPD molecule per nL three positive hits were acquired as displayed by a green checkmark (B). Through implementing calibration curves for all three combinations it was apparent the primary hit demarcated as (2) was a false positive produced by antibody-antibody binding (C).

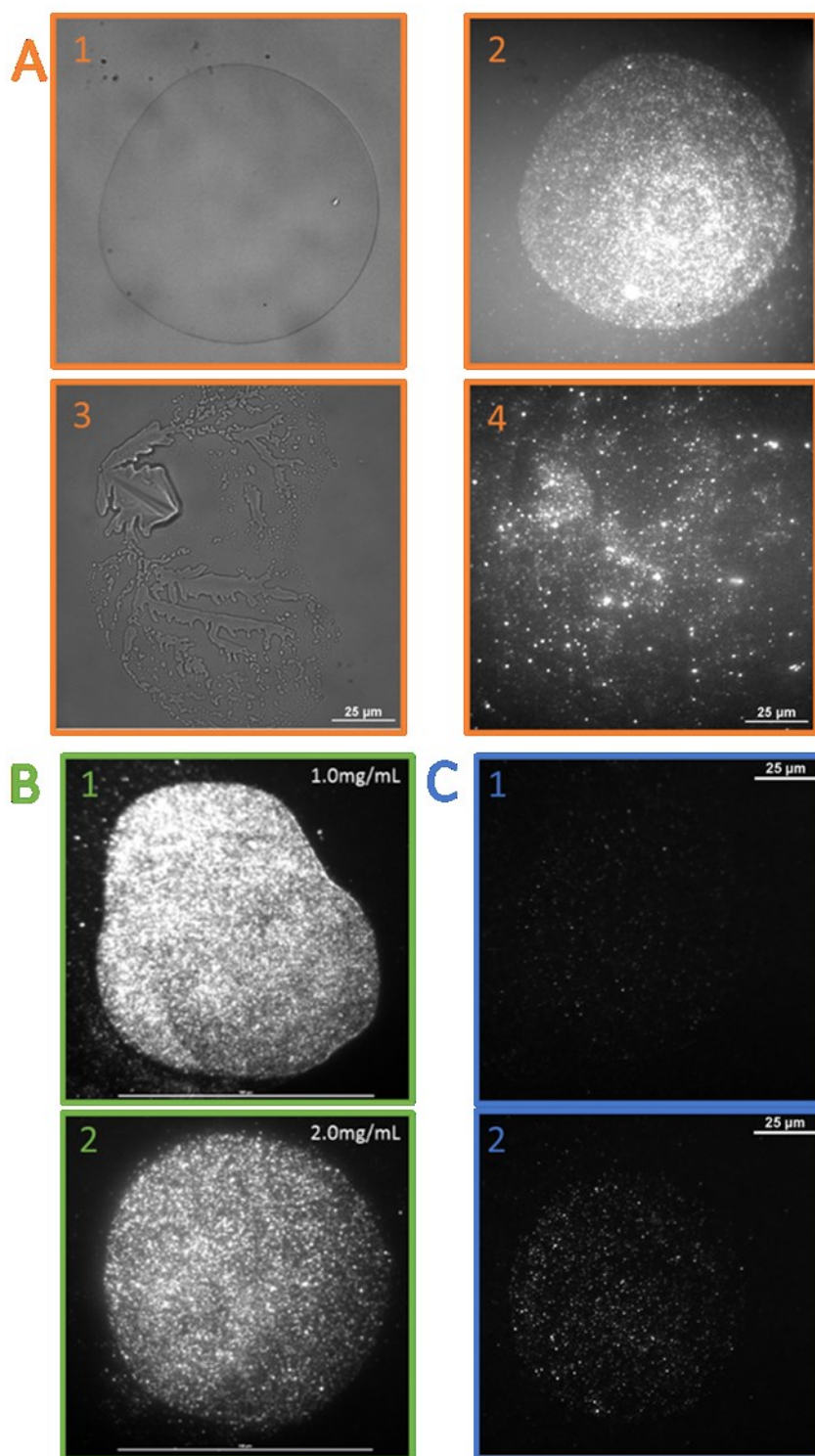


Figure 4.5 Alteration of HPPD-19 Capture Antibody Concentration. To achieve an increase in assay detection multiple methods were attempted to alter the concentration of capture antibody, these raw images are presented. The first method (A) was through alteration of printing buffer, such that in place of the (1) SSC Betaine (3) Array it was implemented. Images are before 60x brightfield and after (2,3) the addition of 1×10^6 His-AtHPPD-delta28 60x TIRF respectively. The second method (B) was through increasing the amount of printed antibody, whereby originally 1 mg mL^{-1} was mixed with 1:1 ratio of printing buffer (0.5 mg mL^{-1}) (1). This was increased to 2 mg mL^{-1} and mixed with 1:1 ratio of printing buffer (1 mg mL^{-1}) (2). This increase appears to have induced a decrease of antigen binding events conducive of steric hinderance. The third and presented a 2-fold benefit was through conjugation of the capture antibody with biotin (C). A concentration of 1×10^4 His-AtHPPD-delta28 was assessed in open chip format for without (1) and with biotin conjugation (2).

4.2.3 Developing the HPPD-19 Assay

The 4-HPPD multimer detection assay, consisting of HPPD-19: HPPD19, while superior in the order of magnitude for detection, contains a flaw, a lack of affinity, such that no binding events are witnessed when 1×10^4 HPPD molecules are present. Elaboration upon affinity, descriptive of the interaction between antigen and antibody have been presented in section 2.3.2. Improvement in antibody detection can be achieved by two ways, increasing either the antibody concentration or the equilibrium constant. While the latter, such as by affinity maturation by alterations in cellular immune response, is unachievable. Therefore, manipulation of antibody concentration was carried out to assess antibody detection.

The first approach in concentration alteration was performed by implementing the Array-it -printing buffer in place of the 3X SSC 1.5M Betaine solution, as previously identified in section 2.3.3, differing printing solutions produce alterations in spot antibody content. In an open chip experiment, as shown in *Figure 4.5A*, it is apparent the additives in the Array-it printing buffer did not present a preferential physiological environment for microarray formation. The second approach, while expensive, was alteration through increasing the directly printed concentration of antibody. Primary antibody was arrayed at 2 mg mL^{-1} while maintaining the same 1:1 ratio with printing buffer. This alteration presented a 9.57-fold decrease in antibody binding, as presented in *Figure 4.5B*. It may be postulated that by printing at a higher antibody concentration, steric hindrance is created. The prevention of antigen binding by neighbouring antibodies. A third approach is creating favourably orientated capture antibodies, where Biotin is conjugated to the F_c region of an antibody, facilitating a higher amount of actively binding antibodies within the printed spot. As presented in *Figure 4.5C*, favourable orientation was beneficial, yet only produced a 2x increase in detection capability. Through implementing this last approach, biotin conjugation upon the HPPD19 capture antibody, the 4-HPPD assay calibration curve was acquired, as displayed in *Figure 4.6 A*.

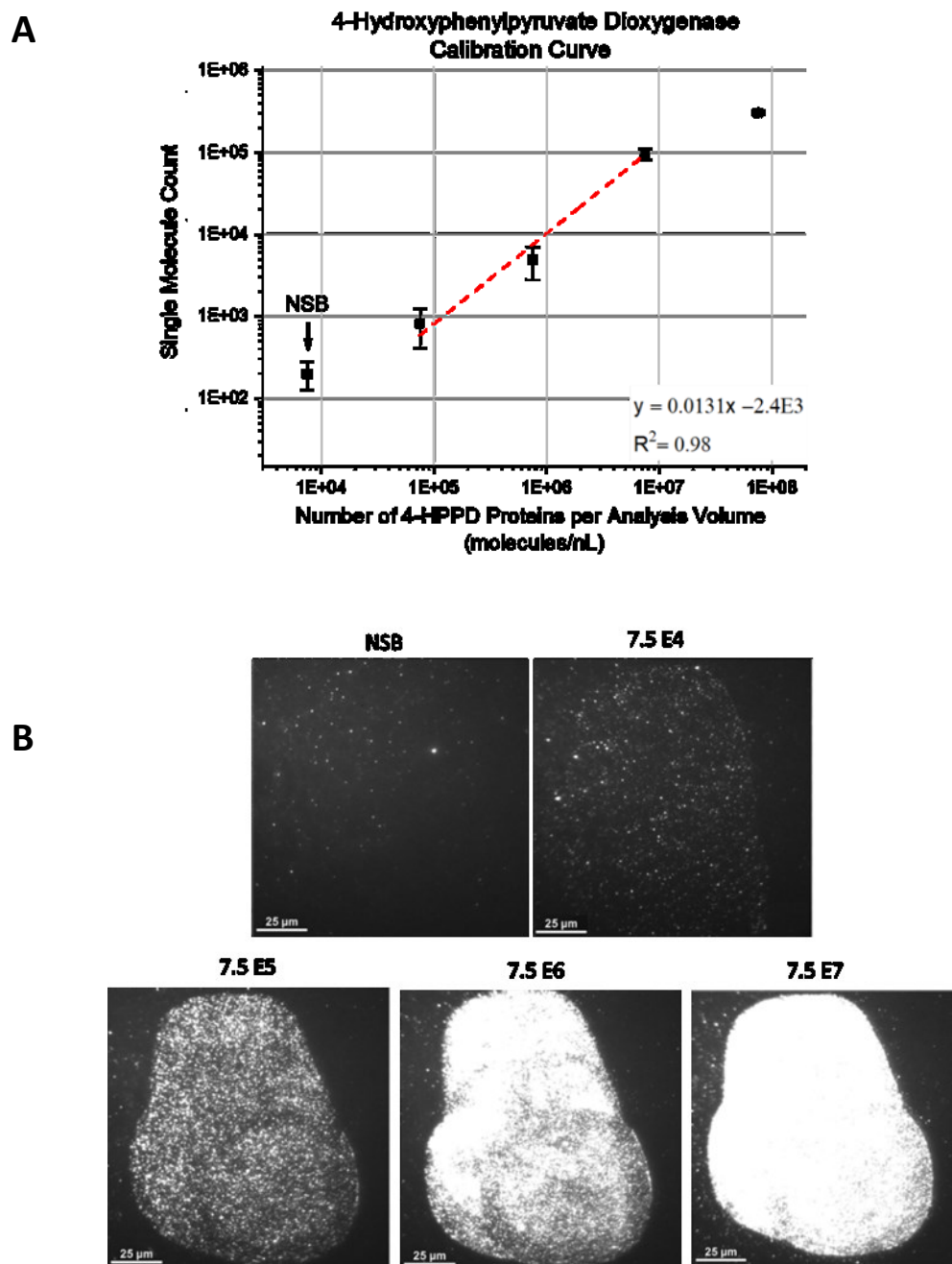


Figure 4.6 Calibration Curves for the 4-HPPD Assay. A known amount of recombinant 4-HPPD is introduced into 0.75 nL microfluidic assay chambers. (A) After equilibrium has been reached ($t=60$ minutes) the tri-molecular bound 4-HPPD, identified by the detection antibody and quantified by single molecule binding events. A linear range ($y = 0.0131x - 2.4E3$) is identified between the 4-HPPD (molecules/nL) introduced into the assay and the binding events measured (single molecule count). The linear range allows a standardized 4-HPPD content to be recognized with only the identification of the single molecules bound at assay equilibrium. (B) Raw images of tri-molecular assay binding from the 0.75 nL chamber 4-HPPD calibration experiment. The number above denotes the 4-HPPD molecules/nL present in the chamber and the LUTs have been scaled identical in each image to allow the identification of non-specific binding single molecules in the image titled NSB.

4.3 Bulk Cell Analysis

The initial approach of single-cell analysis, a rather lofty goal in hindsight, lead to no detection of 4-HPPD. Upon this inability to achieve single-cell assay detection, a multiple cell approach was attempted. Multiple cells ranging from 2-10 were placed inside assay chambers and lysed, with no apparent detection of 4-HPPD. A final attempt was implemented where 1×10^6 cells per mL were lysed through off-chip chemical methods, this lysate was vacuum infiltrated into the chip, and antibody spots were assessed for 4-HPPD binding events.

4.3.1 Open Chip Experiment

While the calibration curve has been produced with recombinant protein, a pre-assessment of this assay with native 4-HPPD was necessary. As *in situ* post-translational modifications or protein-protein interactions may render the protein of interest undetectable through antibody-antigen interactions. Through performing an open chip experiment, whereby 30 μ L of partially lysed isolated chloroplasts resuspended in (4% BSA in CIB with 1×10^7 HPPD-19 conjugated to AlexaFluor 488) was pipetted upon a microarray of HPPD-19 capture antibodies. This preliminary experiment presented an increase in assay binding events as displayed in *Figure 4.7*.

4.3.2 Single Protoplast Measurements

Singe protoplasts were interrogated using the methodology outlined in section 2.8. Single cells were loaded into assay chambers, lysed thereby liberating 4-HPPD, and antibody spots were assessed for single molecule binding events. Non-specific binding events were measured, yet no protein specific binding events took place as presented in *Figure 4.8A*.

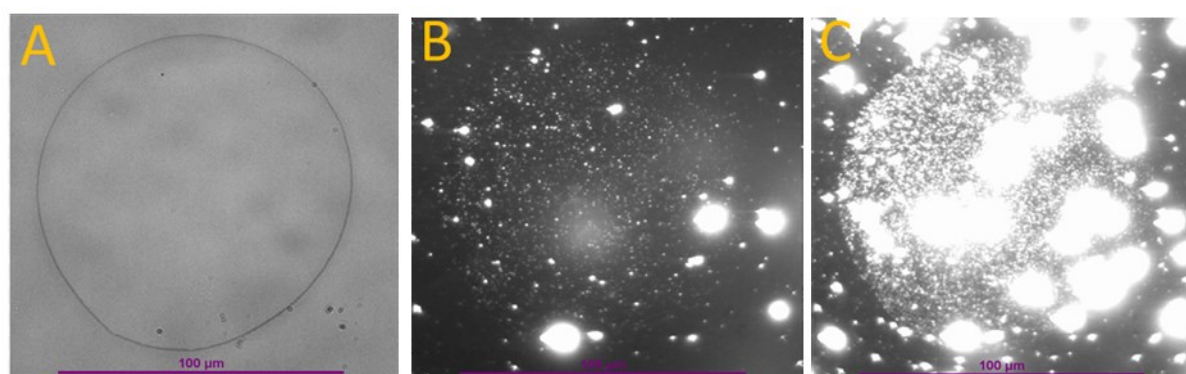


Figure 4.7 Assessment of Chloroplast Lysate for 4-HPPD. The implementation of recombinant protein to produce a calibration curve may not be truly representative of a working assay. As the protein found in lysate may not interact with the assay antibodies due to post-translation modifications or protein-protein interactions. Preliminary assessment of *Spinacia oleracea* chloroplasts partially lysed were added upon microarray SSC Betaine antibody spots, 60x Brightfield (A). Upon assay evaluation at 10 minutes by 60x TIRF image acquisition (B) and 30 minutes (C). An increase in binding signal can be seen below the larger fluorescent objects (intact chloroplasts and chloroplastic thylakoid membranes). Scale bar = 100 μ m

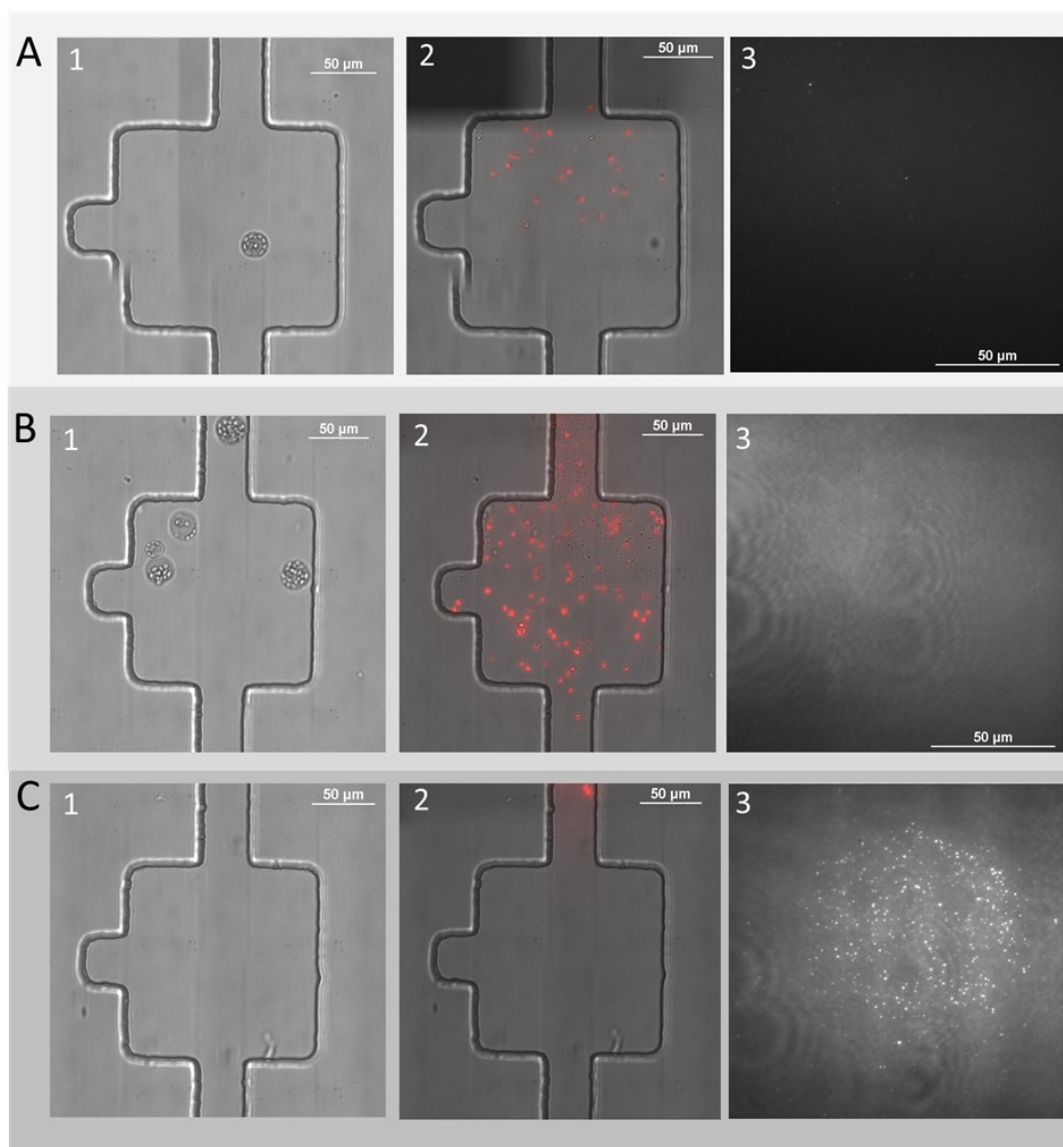


Figure 4.8 Approaches to Identify 4-HPPD Content in Single, Multiple, and Bulk Cell Lysate. (A) Single cells were (1) loaded into assay chambers, underwent (2) optical lysis followed by chemical lysis. (3) The signal generated from the assay was indistinguishable to that of the assay non-specific background (3). (B) Multiples of cells were (1) loaded into assay chambers, after (2) optical and chemical lysis no signal specific to assay binding is present. However, an increase in background from cellular autofluorescence can be detected. A final attempt of infiltrating assay chambers with cellular lysate (100 cells per chamber) presented assay binding, (3) distinguishable by eye and single-molecule localisation assessment from the background of cellular autofluorescence.

4.3.3 Multiple Protoplast Measurements

In an attempt, to assess this assays limit of detection concerning single cell resolved measurements of 4-HPPD content, multiple protoplasts (2-10) were loaded into assay chambers as shown in *Figure 4.8B*. This led to an increase in fluorescence signal not attributable to the NSB of the secondary antibody but to the presence of plant autofluorescence. This contribution of autofluorescence fluorescence signal is negligible in single plant cell studies, as quantified in section 2.8.1, yet appears influential in multiple plant cell measurements.

4.3.4 100 Protoplasts per Analysis Chamber Measurements

To achieve a possible readout signal from this assay it was determined a bulk analysis approach would be deemed appropriate. While a loss of single cell resolution is faulty for assessment of population heterogeneity, this protein has not been quantified. Therefore, bulk protoplast analysis would permit insight towards the average copy number of 4-HPPD. After protoplasting, following the methods outlined in section 2.6.1, protoplast content was quantified through the implementation of a hemocytometer. Protoplasts were pelleted by centrifugation and resuspended in RIPA buffer, following the manufacturer's protocol. Protoplasts were diluted to a concentration of 1×10^6 cells mL^{-1} or 1×10^3 cells μL^{-1} and vacuum infiltrated into MAC Chip analysis chambers, allowing the quantification of an estimated ~ 100 cells per analysis chamber. As shown in *Figure 4.8C*, a signal symbolic of protein specific binding events was acquired.

It has previously been shown that by inducing etiolation of barley leaves, such that plants are placed into darkness for 2 days, an increase in RNA (presenting sequence homology with a gene coding for 4-HPPD) was measured [200]. Measurements of this RNA content was multiplexed with measurements upon SSU (Rubisco Small Subunit) presenting inversely correlated measurements concerning 4-HPPD RNA content, as shown in *Figure 4.2*. Modification of SSU RNA content has previously been identified, in *Nicotiana tabacum*, to be an indication of light conditions, as previously described in Section 3.4.2. To assess possible alterations of 4-HPPD content upon etiolation, protoplasts were isolated from etiolated *Arabidopsis* leaves, visual assessment of leaf viability is displayed in *Figure 4.9*. Protoplasts underwent lysis and vacuum infiltration into MAC Chip Assay chambers following the protocol followed above for healthy *Arabidopsis* leaves.

The resulting measurements of etiolated protoplasts compared to healthy protoplasts are presented in *Figure 4.10*. It can be estimated that upon the induction of etiolation 4-HPPD content per protoplasts elevates by 2.6-fold. Such that, healthy protoplasts contain 271 ± 98 4-HPPD molecules and etiolated protoplasts contain 975 ± 100 4-HPPD molecules.

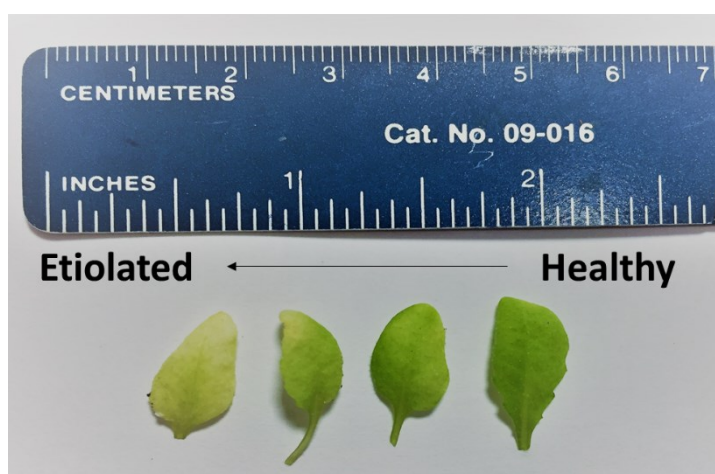


Figure 4.9 Leaf Visualization of the Induction of Etiolation. Through introducing whole *Arabidopsis thaliana* plants into darkness for two days leaf etiolation ensued, descriptive of the yellowing of leaf tissue through alterations in gene expression. Exemplar leaves implemented in multiple protoplast assessment.

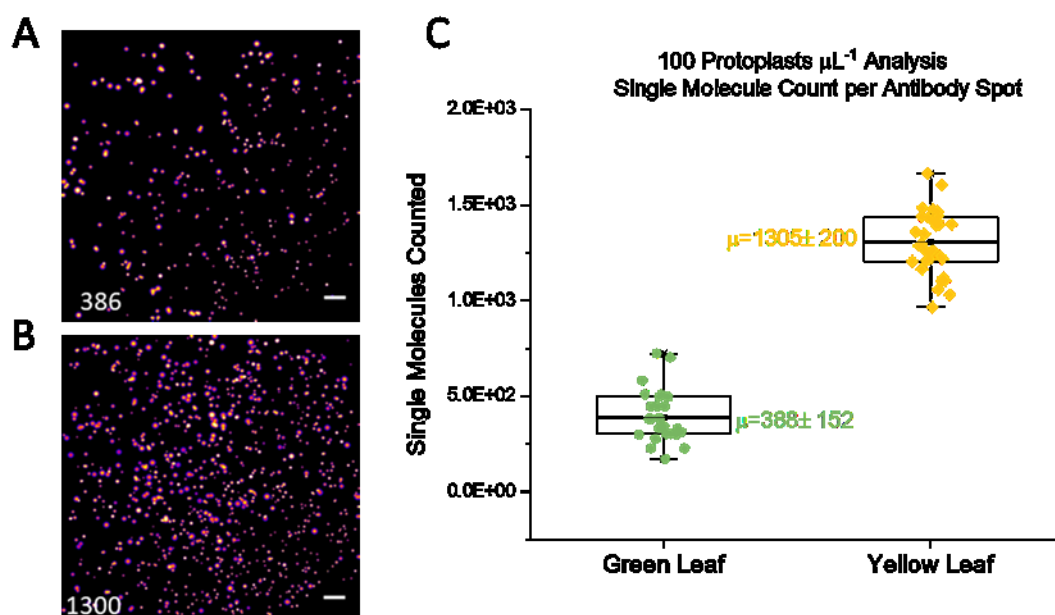


Figure 4.10 Bulk Assessment of 4-HPPD Content in Healthy and Etiolating Leaves. Through bulk cell lysate analysis of 4-HPPD, images of 31 microarray capture antibody spots were evaluated from single molecule localization. Images respective of an average antibody spot of healthy (Green Leaf) (A) and etiolated (Yellow Leaf) (B) experiments, the number of single molecules identified are presented in the bottom left corner. These measurement distributions of antibody spots present similar standard deviations, representative of the reproducibility of antibody printing with an average single molecule count of 388 and 1305 for healthy and etiolated leaves respectively (C).

These values were determined by application of the 4-HPPD calibration curve from the average value of single molecule quantification for the 31 Antibody spots present within each MAC chip experiment. The average measurement was $3.8 \pm 1.5 \times 10^2$ and $1.3 \pm 0.2 \times 10^3$ single molecule binding events respectively. However, the association of the bulk measurement copy numbers with bulk measurements of chlorophyll autofluorescence would be further advantageous for characterising the etiolation status for the population of cells.

4.4 Conclusion & Future Work

By selectively screening multiple antibody combinations through open chip microarray format, an assay for 4-HPPD identification was achieved. As 4-HPPD has not been quantified, through either bulk or single cell analysis methods it was imperative to increase the sensitivity of the assay. This was achieved by alteration to the concentration of capture antibody present within the assay chamber. First, through manipulation of printing buffer, then printing at a higher concentration of capture antibody, and finally by favourable antibody orientation. The latter achieved by the conjugation of biotin to the F_c region of the capture antibody. This presented a 2-fold increase in performance compared to the unmodified assay.

Integration of this assay for single cell analysis presented pitfalls, representative of assay detection limitations. To surpass the limit of detection, multiple cells were loaded into

assay chambers presenting no signal generation. Therefore, bulk cell lysate of 1×10^6 cells/mL was vacuum infiltrated into the assay chambers, showing an average signal for the 31 antibody spots measured corresponding to 271 ± 98 4-HPPD molecules and 975 ± 100 4-HPPD molecules for healthy and etiolated mesophyll protoplast respectively.

This chapter illuminates a small, but imperative problem for single cell detection within the MAC Chip; the variability of antibodies. Surface immobilisation of antibodies comes at the cost of decreased avidity comparative to freely diffusive antibodies. Therefore, the antibody with a higher avidity towards the protein of interest should be immobilised *Figure 4.11*. A majority of antibody manufacturers provide limited data on antibody binding kinetics, while off target antibody-antibody interactions were hard to predict, leading to a high financial cost to advance the MAC Chip technology towards the quantification of new proteins. A novel approach to capturing proteins through DNA interactions or fragmented antibodies may present a beneficial aspect for furthering the MAC Chip technology. However, furthering the technology in this thesis will focus on design alterations by fabrication of closed assay chambers and protein measurements upon a pre-existing p53 and GFP quantification assay involving previously validated antibodies.

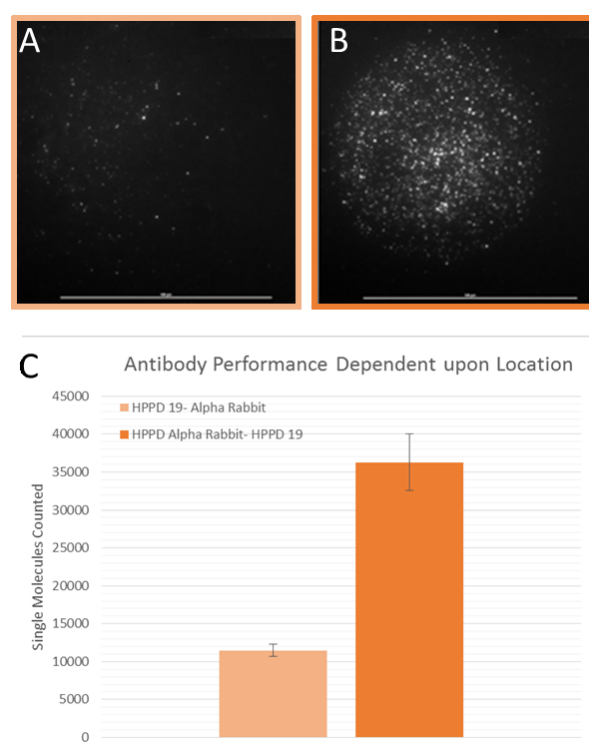


Figure 4.11. Assay Performance Exhibiting Dependence on Antibody Location. As two antibodies are implemented within a tri-molecular assay, two antibody locational environment are possible. One antibody is a capture antibody, immobilized upon the cover glass surface. The other antibody is bound with a fluorophore and diffusive within the assay solution. Locational alterations of the same antibody pair show an increase of single molecule binding events(C). Such that the antibody exhibiting the greatest affinity in the pair when in solution (A) presents lower binding events, comparative to when the antibody is placed as the capture antibody. Providing evidence towards greater assay sensitivity, when the capture antibody exhibits the highest affinity within the antibody pair.

Section 2:

Throughput Promoting Technologies Towards Miniaturised Protein Quantification Assays

Chapter 5: A Reduced Volume Microwell Array

Chapter 6: Quantitative Fluorescence Microscopy

Chapter 5

A Reduced Volume Microwell Array

Optical trapping, to facilitate the isolation of single cells, is tedious and time-consuming. This experimental chapter discusses a novel approach towards integrating the previous miniaturised assay technology into a microwell array format, in an attempt to increase experimental throughput. Initial appraisal of this approach is provided by comparing the number of cells present and the measured assay fluorescence in each microwell. The calibration of a p53 tri-molecular assay follows, presenting both theoretical and experimental evidence that reducing chamber volume and increasing antibody capture spot diameter returns a higher antigen capture percentage. This chapter concludes by combining the novel approach with a p53 tri-molecular assay for the detection of absolute copy numbers for p53 in mammalian cells.

5.1 Introduction

Microwell arrays can be powerful platforms for facilitating biological measurements due to the confining of molecules and cells into small volumes, thereby creating a higher local concentration of the analyte to be measured [202]. Detection advantages provided by volume reduction in combination with digital readout systems have been previously presented by the Hansen and Walt groups allowing detection of extremely small concentrations (sub femtomolar) of analyte in (1-2 pL) microwells. Larger (10-20 μ L) microwell arrays have also provided efficient capture and culture environments for cells and bacteria [203]. Concerning single cell analysis, microwell arrays have also presented to be beneficial for the separation and isolation of single cells from bulk populations, thereby allowing further downstream analysis such as PCR [204]. Through combining the biological measurement advantage and cell capture efficiency of microwells, analogue measurements upon the secretions of human peripheral blood mononuclear cells have been achieved, by integrating the capture reagents (antibodies) into the microwell chambers [105].

The proficiency of implementing microwell arrays for single cell capture is defined by a stochastic nature that can be regulated by three variables. (1) The microarray pattern

dimensions, whereby the aspect ratios of width and depth of the microwell influence the rate of fluid infiltration [205]. The microwell width provides an influence upon another variable (2) the sedimentation time of the organism, such that the inertial forces acting on the cell are functionally dependent upon microwell size [206]. The sedimentation time of organisms located within a microwell is less than an organism located in the bulk solution being administered to the microwells, therefore if the allotted sedimentation time is small, to avoid multiple-organism occupancy, the capture efficiency of organisms will differ consistently with the last variable (3) the cell concentration [207].

Understanding cell heterogeneity is one of many investigations made possible by single-cell analysis, as this approach avoids the loss of information associated with ensemble averaging from bulk cell analysis. One aspect of cell heterogeneity is the protein expression variation, defined as noise as discussed in section 1.5.2; this variation is attributed to stochastic processes such as transcription and translation [208]. This variation in protein expression has been postulated as a resistance factor in cancer progression after drug treatments [166,167]. Furthermore, this variation in protein expression can be a time-dependent dynamic process, such that the oscillation frequency or signal duration provide further cell regulation. An example of a dynamically expressed protein is p53, nicknamed “the guardian of the genome” a tumour suppressor protein, which triggers multiple cellular programs in DNA repair, cell cycle arrest, or terminal fates such as apoptosis [210]. The measurement of p53 expression can be of interest for single cell protein noise measurements as altered levels of expression are characteristic for early detection in breast and gastric cancer [211,212].

This chapter will show comparative analysis between the p53 expression noise of a wild-type p53 expressing cell line (MCF7) and that of a mutated p53 expressing cell line (MDA-MB-231). This analysis is achieved by incorporating the previously described digital readout miniaturised ELISA technology discussed in section 2.3 into microwell arrays, fabricating a novel device titled the Flip Chip.

5.2 Materials and Methods for Microarray Development

5.2.1 Mammalian Cell Culture

Two breast cancer cell lines were utilised to evaluate the performance of the reduced volume microarrays. The MDA-MB-231 (ATCC HTB-26) is a breast adenocarcinoma cancer cell line which has been shown to overexpress a mutant p53, while the MCF7 (ATCC HTB-22) breast

cancer cell lines produce a wild-type p53 which maintains a normal level of expression [213]. A colorectal cancer cell line titled BE (or basal endothelial) was also implemented for analysis of p53 expression. This cell line was donated from collaborators at the Institute for Cancer Research. From our previous p53 analysis, the group has witnessed this cell line to be a highly expressing p53 cell line, yet the mutation status of the p53 is still unknown [214].

5.2.1.1 Cell Culture Methods

All three cell lines were cultured in tissue culture flasks (Corning, UK) using Dulbecco's Modified Eagle Medium (Corning, UK) supplemented with 10% fetal bovine serum (Corning, UK) at 37°C and 10% CO₂ atmosphere. Before experimentation, cells were washed with PBS and then detached from the tissue culture flask using Trypsin-EDTA (Gibco, UK). Cells were resuspended in PBS and pelleted by centrifugation at 200g. The pellet was treated with Hoechst 33342 (Fisher, UK) following the manufacturer's protocol, to produce the DNA intercalating fluorescence signal for cell location identification after seeding cells into the reduced volume microarrays.

5.2.1.2 Cell Size Measurements

To determine accurate pattern dimension for the trapping microarrays, three cell lines were measured for cell size as shown in *Figure 5.1*, by imaging cells deposited upon a coverslip and performing spatial measurements as discussed in section 2.4.4. The MCF7 cell line displayed a maximum cell diameter of 24.76 μm and an average of $15.61 \pm 0.30 \mu\text{m}$. This average is comparable to the BE cell diameter of $15.65 \pm 0.24 \mu\text{m}$ and the MDA-MB-231 cell diameter of $17.27 \pm 0.22 \mu\text{m}$. From these cell size measurements, it was concluded that the trapping microarrays should consist of a 25 μm depth.

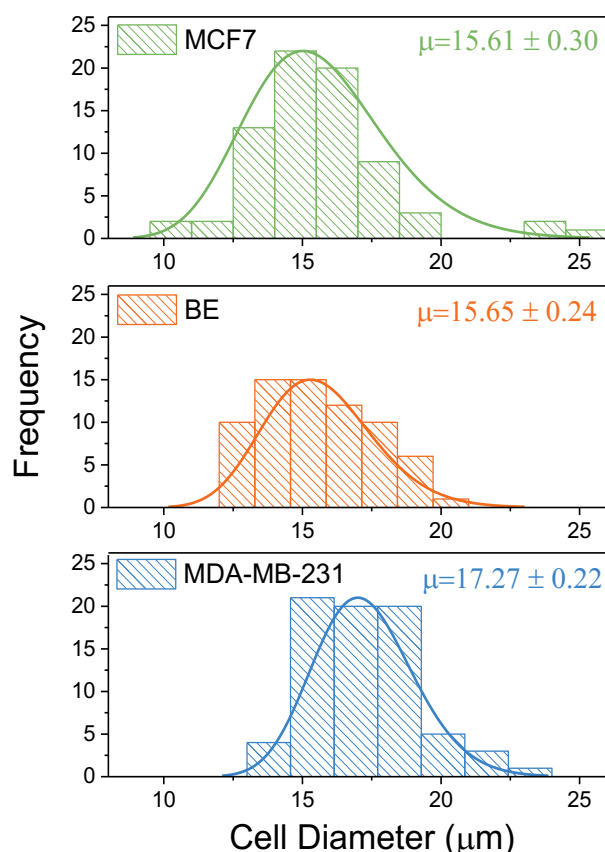


Figure 5.1 Mammalian Cell Size Comparison. To determine an accurate pattern dimension for the cell trapping arrays, cell size was quantified. All three distributions fit best to a lognormal distribution, as validated through an A-D test for Goodness of Fit. The average diameter of an MCF7 cell was 15.61 μm , BE cells were similar with an average diameter of 15.65 μm and the MDA-MB-231 had a larger average cell diameter of 17.27 μm . Yet the MCF7 cells displayed a larger maximum cell diameter of 24.76 μm , this was concluded to be the limit for the depth of the trapping arrays.

5.2.2 Materials and Methods for Microarray Development

Two different soft lithography design approaches were taken to fabricate microarrays for cell trapping which would circumvent the need for optical trapping. These trapping chambers increased the experimental efficiency by increasing the number of cells which can be analysed. Proof of concept experiments for this new microarray device, titled the Flip Chip, was carried out by incorporating a previously validated p53 assay, upon BE cells.

5.2.2.1 Methods for Device Fabrication

The two different designs were both fabricated using established soft lithography procedures, as previously outlined in section 2.2.3, with modifications. Double layer soft-lithography created the first version of the Flip Chip (Flip Chip A), this created a two-chambered microfluidic trap. First SU-8 2002 (Microchem, USA) was spun upon a silicon wafer at a thickness of ~7 microns. After heat treating and ultraviolet light development,

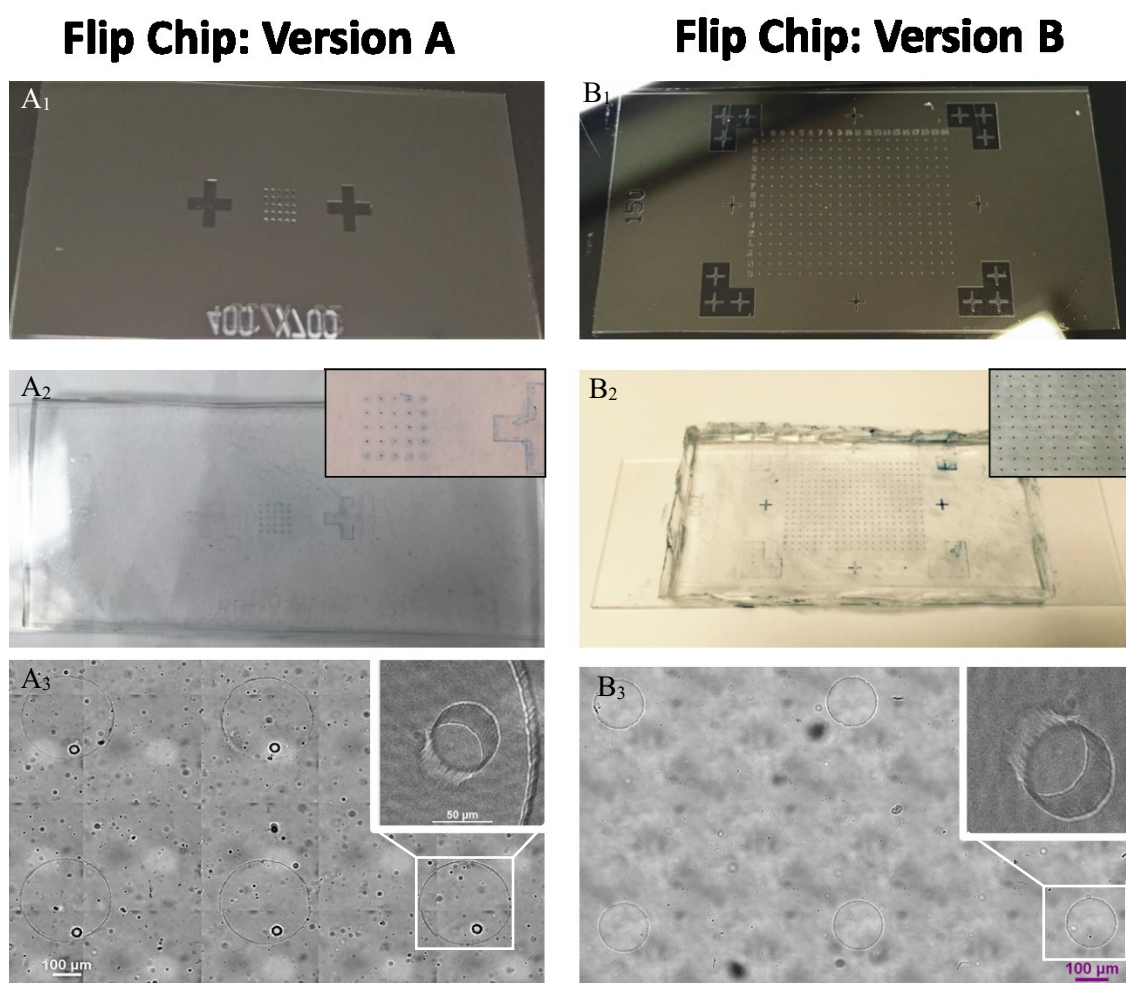


Figure 5.2 Flip Chip Fabrication. Two designs, Flip Chip A and Flip Chip B were fabricated implementing soft lithography procedures, (A₁ and B₁) present the moulds implemented. (A₂ and B₂) After curing a mixture of 5:1 PDMS upon the moulds a microarray device is created, the insert shows the microarrays sealed and filled with trypan blue. A 20X Brightfield image, with inserts of horizontally presented light for contrast, shows the fabricated trapping structures whereby a dual well system is created (A₃) and a single well is created (B₃).

antibody chambers of 300 microns in diameter were created. The unpolymerized photoresist from this layer was not removed, allowing a flat surface for SU-8 2050 to be spun on the wafer at a thickness of ~20 microns. After heat treating and ultraviolet light development of this layer, a cell trapping chamber of 20 microns in diameter and 27 microns in depth was created. The unpolymerized photoresist was removed using SU-8 developer, 2-(-1- methoxy) propyl acetate (Sigma Aldrich, UK), rinsed with isopropyl alcohol and blown dry with nitrogen. A master mould is presented in *Figure 5.2 A*.

The second version of the Flip Chip (Flip Chip B) was created as a single layer by SU-8 2050. Following the standard soft lithography procedures outlined in section 2.2.3 a 150 micron in diameter and 25 microns in depth cell trapping chamber was fabricated, this master mould is presented in *Figure 5.2 B*. Flip Chip A was fabricated only as a simple array of 25 cell trapping chambers, due to alignment complications. Flip Chip B was fabricated as 300 cell trapping chambers. Cell trapping chambers were fabricated in PDMS at a 5:1 w/w mixture of prepolymer and the curing agent. The reason for this modification to 5:1 in place of the previously used 10:1 is to modify Young's modulus of the PDMS. By altering the ratio, as shown by previous work by Liu et al. *Figure 5.3*, the rigidity of the PDMS increases from 610.8 KPa to 1565 KPa [215]. By altering the rigidity of the PDMS, there is a reduced possibility of air becoming trapped within the cell trapping structures. This PDMS is degassed under vacuum and heat cured upon the silicon wafer for 2.5 hours at 65°C.

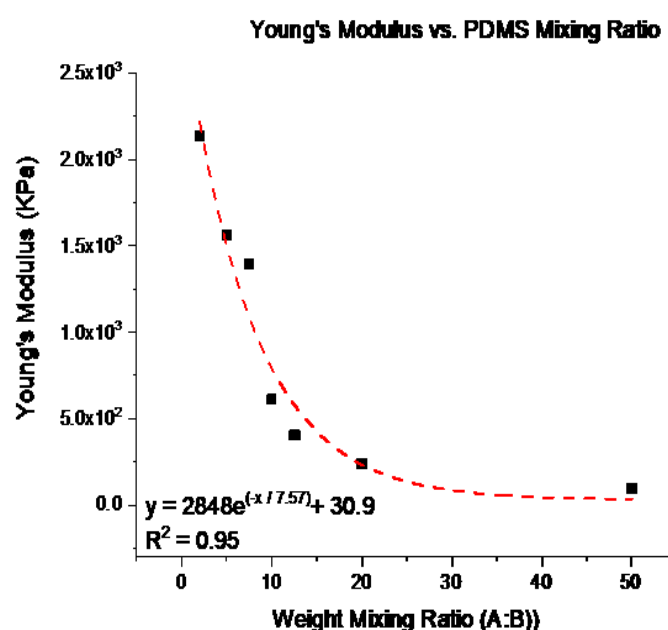


Figure 5.3 Young's Modulus Versus PDMS Mixing Ratio. By altering the ratio of prepolymer and curing agent shown as A and B respectively the Young's modulus may be altered. This relationship can best be defined following the exponential decay equation of ($y = 2848e^{(-x/7.57)} + 30.9$) adapted from Liu et al (215).

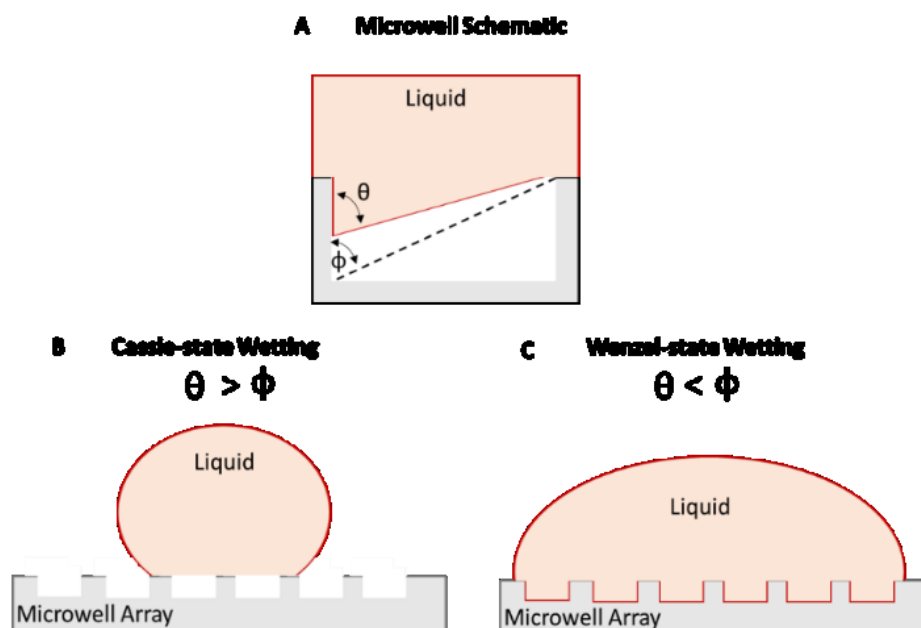


Figure 5.4 Wetting of Microarray. (A) The process of microarray wetting can be described by two angles. θ is the angle of the liquid as it enters the microwell, while ϕ is defined as the diagonal angle from the microwell sidewall into the microwell. (B) When PDMS is in the native form the surface is hydrophobic, liquid will reach the microwell edge before wetting the side and bottom of the well $\theta > \phi$. (C) When PDMS is oxidized by plasma cleaning $\theta < \phi$ therefore air is pushed out from the microwell.

5.2.2.2 Methods of Device Operation

Both designs operate in the same workflow. After the PDMS which contains the microwell arrays has been heat cured for 2.5 hours, the PDMS is removed from the silicon wafer. The PDMS is then plasma cleaned for 2 minutes, producing a hydrophilic PDMS surface which allowed seeding of cells into the microwell array. The motivation for plasma cleaning the PDMS is to introduce a homogenous wetting regime (Wenzel state) such that air cannot become trapped inside the well which would result in heterogenous wetting (Cassie state) which occurs when PDMS is in its native hydrophobic form, as visually portrayed in *Figure 5.4* [216]. Cells were seeded using a previous process described as a wiping method [207], where a coverslip containing 20 μL of cells suspended in (PBS, 4% BSA and 0.5 $\mu\text{g}/\text{mL}$ of secondary antibody) is pipetted along the edge, angled at 45° and slowly wiped across the hydrophilic PDMS microwell array seen in *Figure 5.6*. In Flip Chip A, due to the shallow antibody chamber, cells were only trapped within the cell trapping chamber presented in *Figure 5.6*. In version B cell trapping can occur anywhere within the single chamber, yet the number of single cell isolation events can be manipulated by the cell concentration in both versions of the Flip Chip.

After cell seeding, a coverslip with the capture antibody is placed on top, to seal the flip chip. This coverslip is cleaned, and the surface is passivated following the same protocols discussed in section 2.3.1. However, in Flip Chip A antibody capture spots were printed and

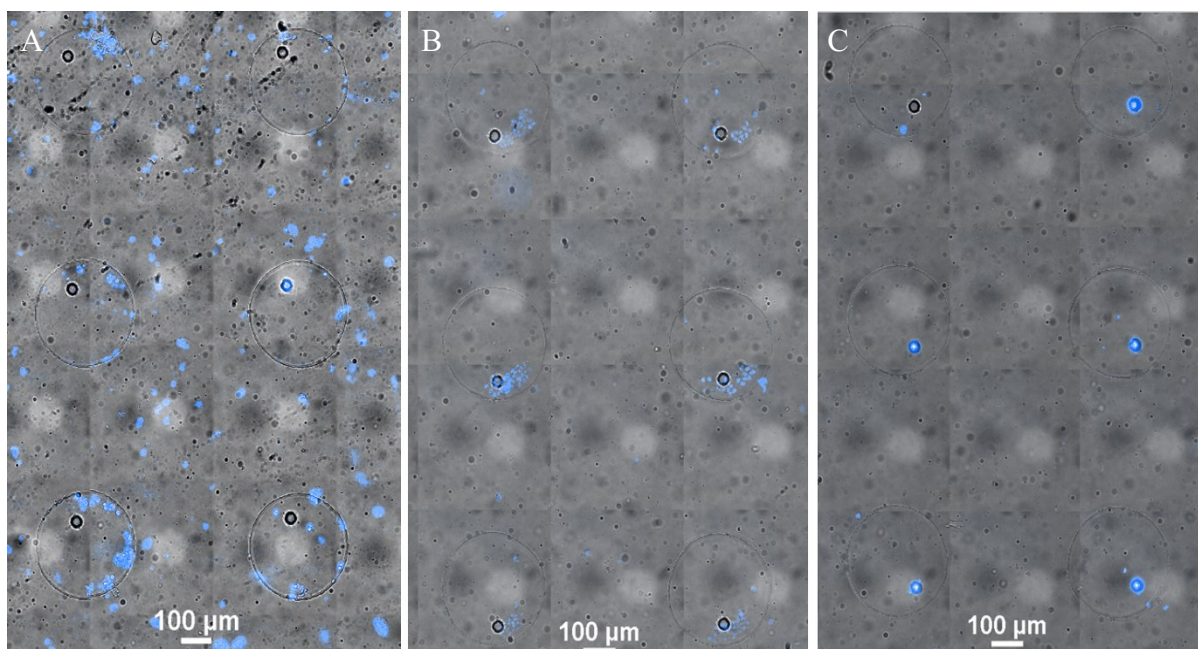


Figure 5.6 Single Cell Capture Efficiency. To accomplish single cell capture within the Flip Chip A, MCF7 cells were fluorescently labelled with Hoechst 3342, demarcated as blue in the above images. Through comparing the methodology of (A) cell sedimentation (the approach A in Figure 5.5) and (B) wiping method (the approach B in Figure 5.6). It can be visually identified that implementing the wiping method achieved a higher accuracy in cell seeding within the microarray. (C) Through decreasing the previous cell concentration to $\sim 1 \times 10^5$ cells/mL the number of single cell capture events increase.

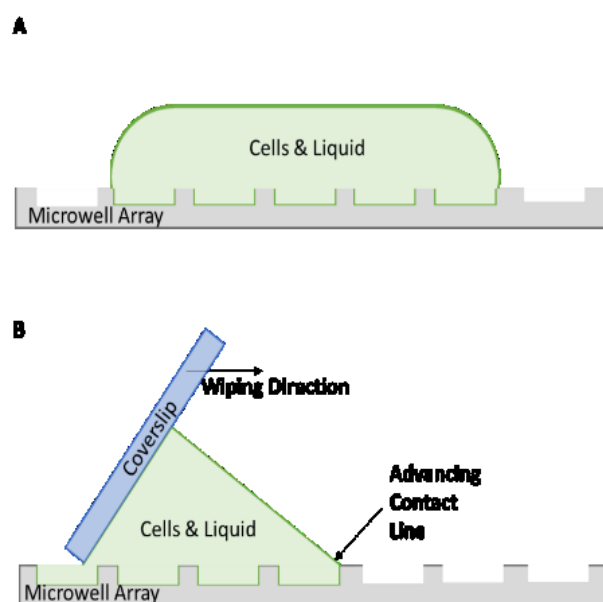


Figure 5.5 Cell Seeding. Two different approaches were taken to seed cells into the microwells. (A) The first approach entails pipetting the cell solution on top of the hydrophillic microwell array. (B) The second approached involves wiping a coverslip at a 45° angle thereby promoting an advancing contact line promoting empty microwells to draw fluid and cells within.

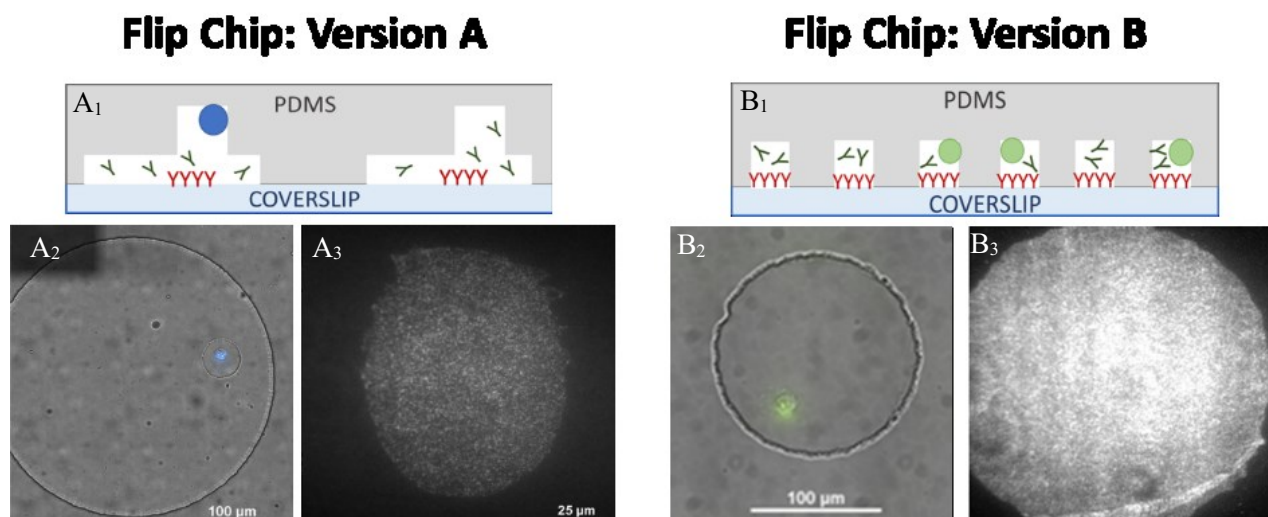


Figure 5.7 Integration of A Capture Antibody Spot into the Flip Chip. A schematic illustration presents both versions of the Flip Chip prior to cell lysis, whereby the ~100 μm capture antibody spot (Y) is aligned into the chamber (A_1) or the 150 μm antibody spot is actualized by confinement within the microwells (B_1). 60X Brightfield microscopy and fluorescence imaging allows cells to be identified as trapped either in the capture chamber of the dual chamber Flip Chip A (A_2) or in the single 150 μm diameter chamber (B_2). After cell lysis the protein of interest (p53) is released and binds to the capture antibody spot and to a fluorophore conjugated secondary antibody (Y). At assay equilibrium the trimolecular species of capture antibody, p53 and fluorescent secondary antibody is illuminated by TIRF microscopy (A_3 and B_3).

aligned within the chambers similar to that of the MAC chip, shown in *Figure 5.7 A₃*. While in Flip Chip B, to overcome alignment complications, the entire coverslip is coated in capture antibody, which has been biotin conjugated. The biotin-conjugated capture antibody is pipetted and sandwiched between two coverslips at a concentration of 0.5 mg/mL. The excess capture antibody was washed with 4% BSA in PBS, yet due to the interactions between the neutravidin passivated coverslip and the biotin-conjugated capture antibody a layer of capture antibody remains upon the coverslip. Once sealed the entire cell trapping well diameter of 150 microns becomes the capture antibody spot, seen in *Figure 5.7 B₃* for the Flip Chip B.

As previously discussed in section 2.7.2, the optical lysis techniques were employed for mammalian cell lysis, an image after lysis can be seen in *Figure 5.8*. Both the 100 μm antibody spot (Flip Chip A) or the 150 μm antibody spot (Flip Chip B), were imaged by TIRF microscopy for tri-molecular binding events a method previously detailed in section 2.4.

5.2.3 Tri-molecular p53 Assay

The assay implemented for p53 detection has been validated by native western blot in previous publications [214]. The implemented assay consists of a printed anti-mouse p53 capture antibody (Enzo Life Science, UK) and a secondary detection antibody D0-1 clone anti-p53 which was labelled with either AlexaFluor 488 or AlexaFluor 647 (Santa Cruz, USA).

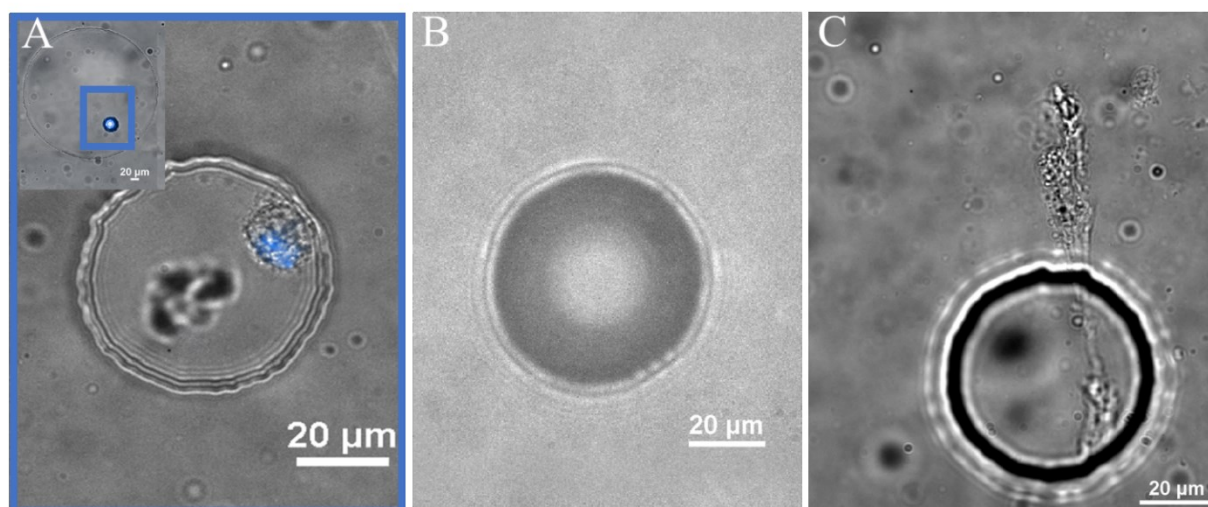


Figure 5.8 Single Cell Lysis in Microwell. (A) After cells are trapped and sealed within the microwell lysis is accomplished by implementing the section 2.7.2 methodology of optical lysis. (B) A cavitation bubble is created within the capture chamber through a 9-ns pulse from a Nd: YAG Laser as shown by a 60X Brightfield image. (C) Upon expansion and the following collapse of the bubble the cell undergoes lysis through shear stress, thereby rupturing the membrane and liberating the intercellular content.

5.3 Flip Chip - Preliminary Assay Implementation

To validate the new methodology of Flip Chip A, BE cells were investigated for p53 content. The tri-molecular assay for p53 detection was utilised in Flip Chip A; this assay has previously been implemented in the MAC chip to investigate p53 copy number in both BE and MCF7 cells [214]. The results from this experiment (n=20 chambers) provide evidence that the p53 signal of binding events increases linearly with the number of cells lysed in each chamber, presented in *Figure 5.9*. This linear agreement provides evidence that there is little to no experimental crosstalk respective of the individual chambers. Furthermore, chambers without cells provided a control antibody spot to determine the background p53 content within the media and the amount of crosstalk between the secondary and capture antibody as previously defined as non-specific background. These control chambers provide a comparable background for subtraction.

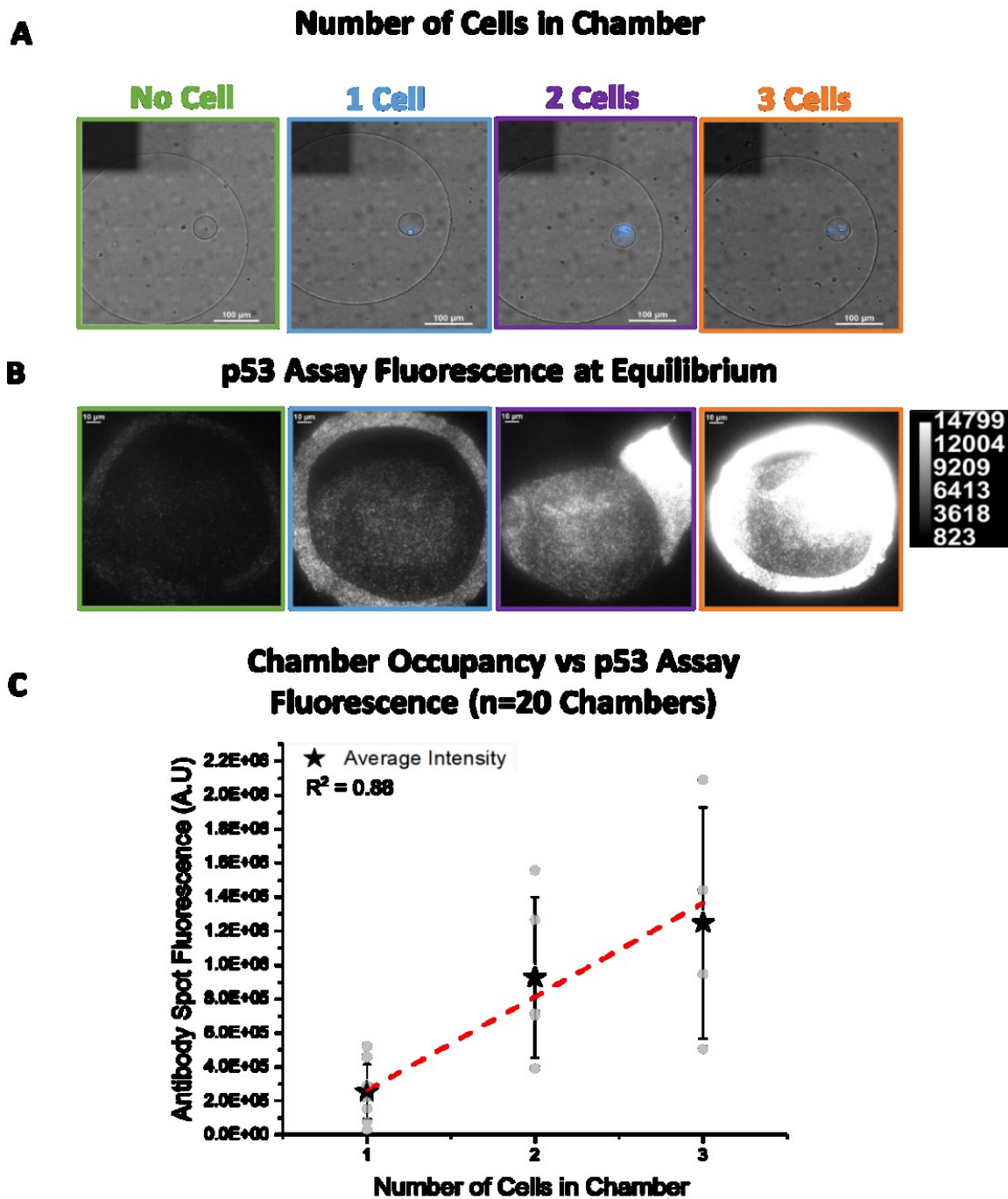


Figure 5.9 Preliminary Flip Chip p53 Assay. Upon loading cells into the Flip Chip chambers a Poisson distribution of occupancy occurs. (A) some chambers contain zero, one, two or three cells, shown as Hoechst 3342 labeled BE cells (blue) in the 20x tiled brightfield image. After cell lysis and the subsequent release of p53, the antibody assay captures p53 denoted as an increase in fluorescence.(B) Images of the antibody spots at equilibrium, the surrounding colour denotation corresponds to the previous brightfield images in (A) . The results as shown in (C) provides evidence that through measuring the linear regression with a y-intercept of 0, the average assay signal (denoted as a black star) is linear with respect to the number of cells lysed ($R^2=0.88$) for the 20 chambers analyzed.

5.4 p53 Assay Calibration

Calibration curves are necessary to determine the absolute amount of p53 released into the microarray trapping chambers after mammalian cell lysis. Calibration curves were created by introducing a known amount of protein into the chambers producing a direct digital readout upon the antibody spot housed within the chamber. To outline the processes undertaken, the p53 assay was calibrated in the 1.75 nL Flip Chip B device. It was also recalibrated in 4.5 nL MAC chip chambers by Vanessa Ho, under my assistance, due to our technological advancements in single molecule identification, as addressed in section 2.4.3. This allowed us to quantify the dissociation constant of the tri-molecular assay (K_D). A theoretical approach may be taken after determining the K_D of the assay to understand how a decrease in volume directly impacts the percentage of binding events witnessed allowing a comparison of theoretical and experimental data within the MAC chip assay chambers and the Flip Chip B confined volume assay chambers. Performing these calibration experiments, have allowed an evaluation on the impact of assay binding dynamics with respect to the choice of conjugated fluorophore on the secondary antibody.

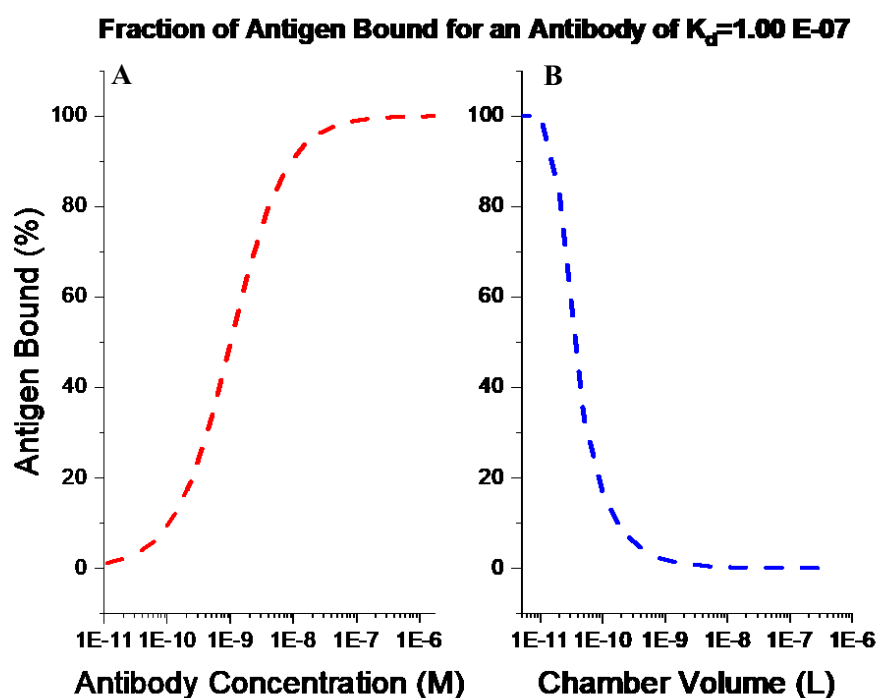


Figure 5.10 Ambient Analyte to Mass Sensing Regime. It is advantageous to operate in the mass sensing regime, whereby the majority of antigen is bound as opposed to the ambient analyte regime where a small percentage of analyte is bound. To motivate an assay into the mass sensing regime a higher antibody concentration, (A) specifically a greater number of available capture antibodies can be introduced into the assay. (B) Alternatively, antibody concentration can be increased through decreasing the assay volume.

5.4.1 4.5nL Chamber Assay Calibration

The dissociation constant of a chosen assay is a crucial starting aspect, concerning the MAC Chip and the Flip chip technology, such that if an assay has a weak affinity (large K_D) for the antigen of interest alterations towards the technology to produce an increase in binding events is unbeneficial. However, if an assay has a strong affinity (small K_D) alterations in chamber volume or printing methodology can transform an ambient analyte regime assay into a mass sensing regime assay. As theoretically presented in *Figure 5.10*, based on the binding kinetics provided in section 2.3.2 for an antibody with a dissociation constant of 1×10^{-7} . *Equation 5.1* has been used to theoretically predict the percentage of antigen bound (B_{Ag}) when the concentration of capture antibody (Ab) is modified, and *Equation 5.2* predicts the percentage of antigen bound when chamber volume (V) is altered with respect to antigen concentration (Ag).

$$B_{Ag} = \frac{Ab}{Ab + K_d} \times 100 \quad (5.1)$$

$$B_{Ag} = \frac{(Ag)(Ab)}{K_d (6.023 \times 10^{23})(V)(Ag)} \times 100 \quad (5.2)$$

To determine the impact of alterations upon the p53 assay, a calibration curve to assess the K_D of the assay was performed, similar to the calibration curve technique performed for Rubisco in Chapter 3, section 3.3.6. A known concentration of p53 is added into 4.5nL MAC chip analysis chambers and the digital readout response of binding events was identified, as presented in *Figure 5.11*. The saturation point, where the linear output of the p53 assay concludes, has been experimentally shown to be reached when 4.5×10^7 to 4.5×10^8 p53 molecules are present within the chamber. With this knowledge, the K_D of the p53 tri-molecular assay can be estimated from a saturation binding curve *Figure 5.13*. Through *Equation 5.3*, which is the equation plotted for the saturation binding curve, estimating the K_D of the assay when B_{Ag} is equal to 0.5 [217]. We show that the K_D of the p53 assay is equal to 1.07×10^{-8} (M) when using the AF488-DO1 secondary antibody.

$$B_{Ag} = 0.5 = \frac{Ag}{Ag + K_d} \quad (5.3)$$

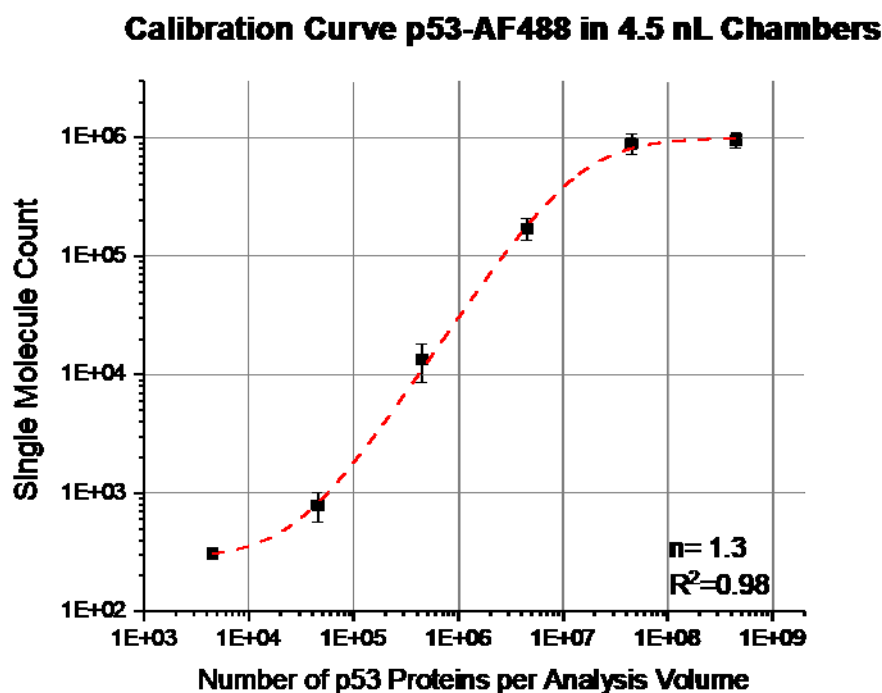


Figure 5.11 A p53 Calibration Curve for AF-488 in 4.5nL Chambers. A know amount of p53 is introduced into the 4.5nL analysis chambers. After the assay has reached equilibrium the bound p53 is quantified. The assay can best be characterized by a Hill fit ($R^2 = 0.98$) whereby the Hill coefficient ($n=1.3$) provides evidence of completely independent assay binding. An assay saturation point, such that a linear response concludes, can be identified between 4.5×10^7 and 4.5×10^8 .

5.4.2 Fluorophore Choice and Antibody Affinity

The ability to achieve multiplexed protein detection from a single cell in a miniaturised ELISA format is reliant upon the ability to conjugate secondary antibodies with fluorophores of different wavelengths respective towards the proteins of interest. From previous research, the concept that conjugating a fluorophore can produce deleterious effects on an antibodies affinity has already been investigated [218]. However, this can still be investigated with specific reference towards the p53 assay. SPR bimolecular assay measurements obtained from Dr. James Nobel at the National Physical Laboratory has shown a 1.78-fold decrease in the DO-1 antibody affinity after the conjugation of the AF 488 and an 11-fold decrease after the conjugation of AF 647, see *Figure 5.12*. These measurements present possible implications with regards to the choice of the fluorophore when designing an assay, such that modifying antibodies can have a dramatic effect on the percentage of analyte bound.

Antibody	k_a (1/Ms)	k_d (1/s)	KD (M)
DO-1	3.37E+04	1.78E-05	5.29E-10
DO-1 488	2.37E+04	3.48E-05	1.47E-09
DO-1 647	1.76E+04	1.09E-04	6.18E-09

Figure 5.12 SPR Antibody Affinity Measurements. Measurements obtained by Dr. James Nobel from the National Physical Laboratory using a Biacore Surface Plasmon Resonance (SPR) Instrument. This provides insight towards the affinity of the non-conjugated antibody (DO-1) in contrast to the same antibody (DO-1) with either a (AF488) fluorophore or (AF647) fluorophore conjugated.

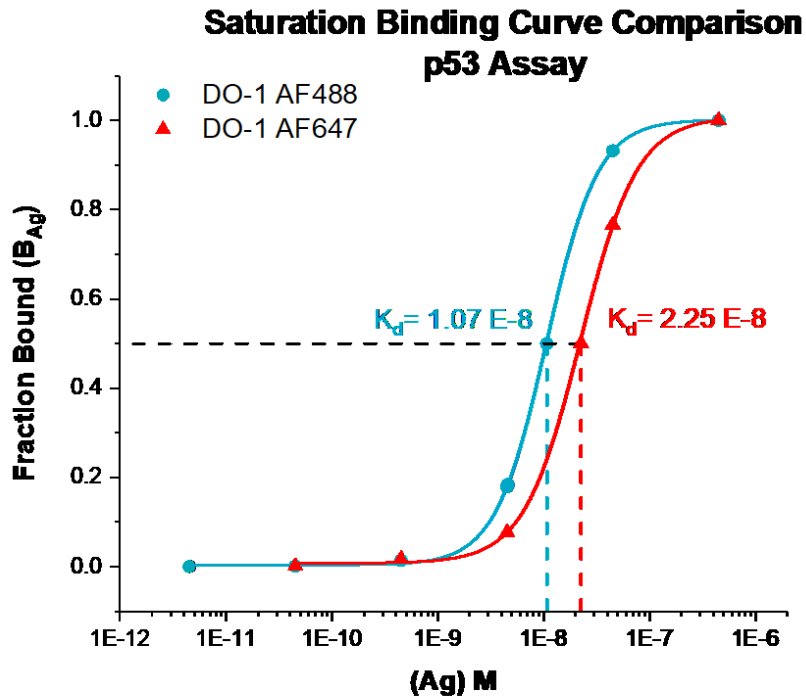


Figure 5.13 Comparison of DO-1 488 and DO-1 647 p53 Tri-molecular Assay Affinity. To determine the K_D of the tri-molecular p53 assay when implementing DO-1 647 as the secondary detection antibody a calibration curve was created as the previously described methods for the DO-1 488. From this a saturation binding curve can be calculated presenting a K_D value of 2.25×10^{-8} for the tri-molecular DO-1 647 p53 assay.

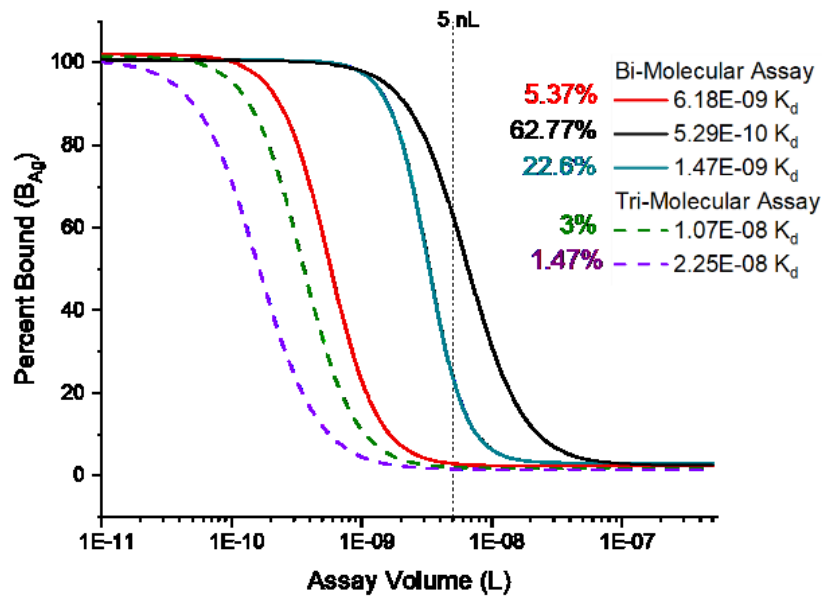


Figure 5.14 Theoretical Antigen Binding with Respect to Measured K_d . Ideal antigen binding with changing assay volumes, based upon the bi-molecular assay (SPR measurements) or tri-molecular assay (MAC Chip measurements) experimentally measured dissociation constant (K_D). In a 5 nL assay volume there is a 3.2-fold difference in the bi-molecular assay and only a 1.1-fold difference in the tri-molecular assay when implementing AF488 compared to AF647.

A theoretical example of these deleterious effects is presented in *Figure 5.14*. By employing *Equation 5.2* and using the SPR measured K_D values we can model in a bi-molecular assay in an assay volume of 5 nL the non-conjugated DO-1 antibody would bind 62.77%. While the DO-1 AF488 would bind 22.6% and the DO-1 AF647 would bind 5.37% of the antigen. However, as previously discussed in the conclusion of Chapter 4, *Figure 4.11*, when creating a miniaturised ELISA the percentage bound (B_{Ag}) of the tri-molecular assay shows a higher dependence upon the affinity of the capture antibody, not the secondary antibody. To further provide experimental evidence of this capture antibody dependence a calibration curve using DO-1 AF647 was measured in the same manner as that of the DO-1 AF488 shown in *Figure 5.13*. By applying the previously discussed theoretical example and applying the measured K_D values for the trimolecular assay, we see that in a volume of 5 nL the DO-1 AF488 would bind 3% and the DO-1 AF647 would bind 1.47%.

In conclusion, while there is a 3.2-fold difference in the measured affinity between the secondary antibody when either conjugated with AF488 or AF647, there is only a 1.1-fold difference in the measured affinity for the tri-molecular assay shown in *Figure 5.14*, providing evidence that the choice of capture antibody and its affinity towards the antigen is the dominating effect in assay detection. After considering this evidence, DO-1 AF488 was chosen for p53 detection for further single cell analysis.

5.4.3 Flip Chip Assay Calibration

In comparison to the predecessor MAC chip, the advantages of implementing the Flip Chip B were two-fold. The volume in the assay chambers has been reduced from 4.5 nL to 1.75 nL while the antibody spot diameter has increased from 100 μm to 150 μm . These two alterations culminate in an increase of 4.8×10^{-9} in the molarity of capture antibodies present (if antibody size is the theoretical limit of antibody packing into spot area) [118]. As shown in *Figure 5.15* the Flip Chip B calibration curve shows an average capture efficiency of 22.35%; this is a $\sim 20\%$ increase in binding efficiency in comparison to the $\sim 4\%$ capture efficiency in the 4.5nL MAC chip chambers. A power function of ($y = 0.4387 x^{0.9383}$) and ($y = 0.803x^{0.9261}$) can be used to respectively describe the 1.75 nL and 4.5 nL trend in assay response. Furthermore, due to an increase of available capture antibody within the Flip Chip B 150 μm antibody spot the saturation point rises to 4×10^6 p53 molecules bound.

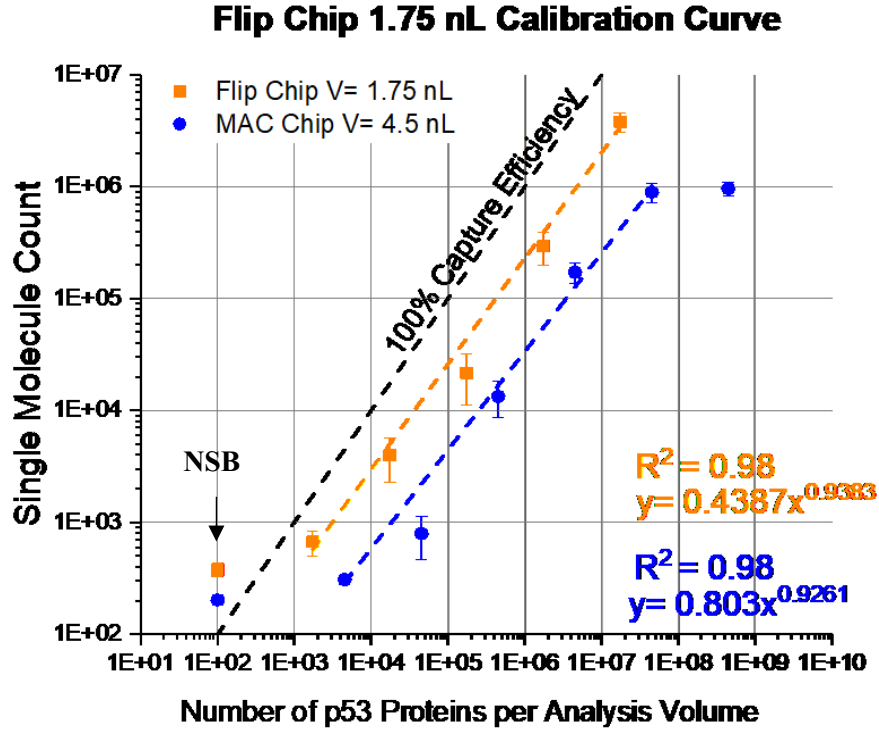


Figure 5.15 Flip Chip B 1.75nL Assay Chamber Calibration Curve. A know amount of p53 is introduced into the Flip Chip assay chambers. After equilibrium has been reached ($t = 60$ minutes) the bound p53 is quantified. The linear region of the assay can be fit to a power function of ($y = 0.439x^{0.938}$) and ($y = 0.803x^{0.926}$) for 1.75nL and 4.5nL assay chambers respectively. This fit allows a standardized absolute p53 content to be recognized with only the identification of the single molecules bound at assay equilibrium. NSB, non-specific background of assay.

Upon experimental evaluation, shown in *Figure 5.16*, with previous MAC Chip calibration curves performed in 9.8nL and 0.2 nL chambers and the previously described 4.5nL MAC Chip calibration curve implementing the DO-1 AF488 as a secondary antibody, *Equation 5.2* can be implemented to model the antigen binding percentage with an 81.84% prediction accuracy calculated from *Equation 5.4*. By increasing the molarity of capture antibody present in the chamber we provide evidence that a 1.75 nL Flip Chip B assay chamber can achieve binding affinities similar to that of 0.75 nL MAC Chip assay chamber.

$$\% \text{ Disagreement} = \frac{\text{Measured} - \text{Predicted}}{\text{Measured}} \times 100 \quad (5.4)$$

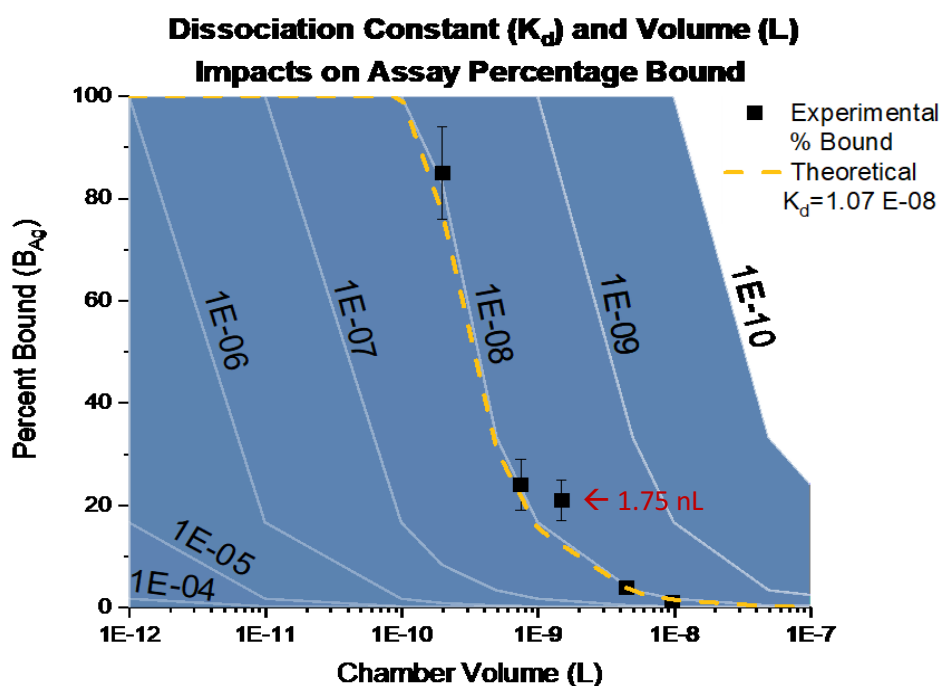


Figure 5.16 Comparison of Percent Antigen Bound for All p53 Assay Volumes Measured. As the affinity of the assay is increased (decrease in K_D), as identified by the gradient moving from dark to light, a greater percentage bound is measured for set volumes (L). By plotting our previous measured binding percentages (0.75nL and 4.5nL) and two previous measured binding percentages (9.8nL and 0.2nL) with respect to assay volume (black square) adapted from [171]. An agreement is shown with the model curve from Equation 5.2. The 1.75nL assay volume does not agree with the model curve as there are a greater number of capture antigens present in Flip Chip B. However, upon comparison a 1.75 nL volume can achieve a similar percent antigen bound to a 0.75 nL when a greater number of capture antigens are present.

5.5 Implementing Flip Chip B for Single Cell p53 Expression

By employing Flip Chip B, we first determined the single cell capture efficiency of the device. After concluding that 3.2×10^5 cells/mL was an optimal seeding concentration, the device was implemented in separate experiments to quantify single cell p53 expression in MCF7 and MDA-MB-231 cells. Quantification of the p53 content was determined by the previous calibration curve ascertained in Flip Chip B 1.75 nL chambers. By performing this experiment on absolute p53 expression, we can start addressing the question of what amount of protein expression noise is tolerated by wild-type cells in comparison to pathological cells.

5.5.1 Single-Cell Capture Efficiency

Three different cell concentrations were implemented to assess the concentration necessary to obtain single cell seeding efficiencies in the Version B flip chip. Following the methods outlined in 5.2.2.2 and keeping the speed of wiping constant at 1.0mm/s. These three chosen concentrations were based on previous optimised single cell seeding experiments which provided evidence that implementing 0.5-2 million cells/mL would be desirable [206,207]. As shown in Figure 5.17 seeding at a concentration of 8.2×10^5 cells/mL led to fewer chambers

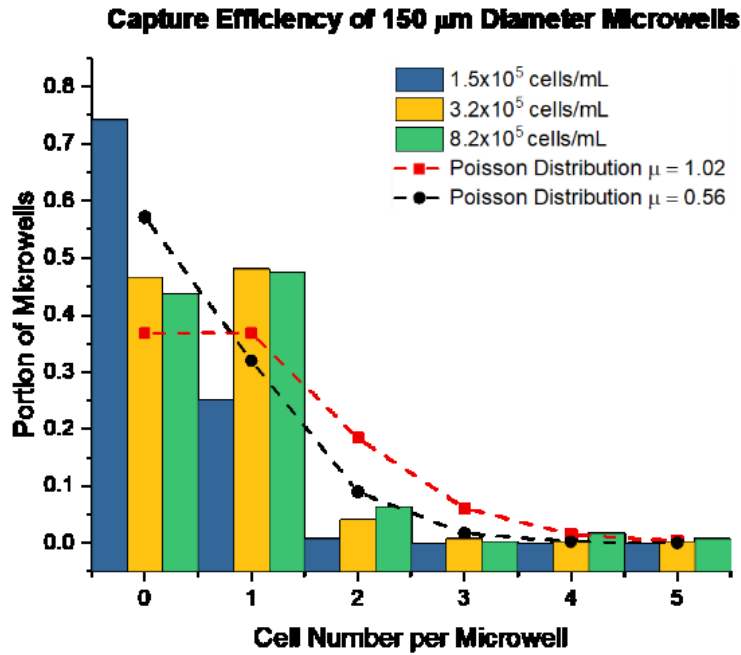


Figure 5.17 Flip Chip B Cell Capture Efficiency. The stochastic nature of cell capture events can be identified as analogous to that of the average rate of occurrence in a Poisson distribution as presented in Equation 1.12. Cell capture events can be defined as the proportion of microwells identified by the number of cells captured for the three measured concentrations of 1.5×10^5 , 3.2×10^5 , and 8.2×10^5 cells/mL. As the concentration of cells increase the probability distribution of the Poisson random variable alters such that the mean value increases from 0.56 to 1.02.

without cells, yet there was a higher frequency of non-single cell seeding events. While seeding at 1.5×10^5 cells/mL presents a higher frequency of chambers without cells. Therefore 3.2×10^5 cells/mL provided an optimum amount of single cell seeding events while avoiding empty chambers or chambers with more than one cell seeding event. However, the preliminary cell seeding results failed to account for the increase of assay background due to the existence of p53 from ruptured cells within the loading media. Therefore, a concentration $\geq 1.5 \times 10^5$ cells/mL was implemented as this produced a binding background before cell lysis comparable with our 1.75nL Flip Chip calibration curve.

5.5.2 MCF7 & MDA-MB-231 p53 Expression Flip Chip B Measured Cells

Implementing previously described methods in section 5.2, single cells were isolated, lysed and quantified for p53 binding events. Binding events were translated into absolute p53 copy number, from the 1.75nL Flip Chip B calibration curve ($y = 0.4387 x^{0.9383}$). The wild-type p53 expressing cell line, MCF7 cells ($n=63$), produced an average p53 copy number of $7.3 \times 10^3 \pm 547$. In a separate Flip Chip B experiment, the mutant p53 expressing cell line MDA-MB-231 cells ($n=56$) were measured showing an average p53 copy number of $5.6 \times 10^5 \pm 1.03 \times 10^5$. The measured cells are shown in Figure 5.18. An initial comparison of the two cell types yields an initial understanding that a pathological (mutant p53) breast cancer cell can

produce 76 times more p53 with respect to a physiological (wild-type p53) breast cell. This increase in protein expression is accompanied by an increase in expression noise (σ^2/μ^2) from 0.35 to 1.88.

5.5.3 Comparison of p53 Expression in 4.5nL MAC Chip Measured Cells

Comparing the increase of p53 expression noise measured in the MDA-MB-231 breast cancer cells with other p53 expressing cell lines, with postulated p53 regulation, one may ascertain an increased understanding of pathogenic cell alterations in p53 gene expression. For comparison, the aforementioned BE cell line and MCF7 cells treated with Nutlin were quantified for single molecule binding events from data obtained by Vanessa Ho under the supervision of Pashiini Supramaniam and Dr. James Squires respectively [220]. These single molecule binding events were translated into absolute p53 copy numbers, presented in *Figure 5.19 A*, from the aforementioned 4.5 nL MAC Chip calibration curve ($y = 0.803x^{0.9261}$).

Alterations in p53 expression noise for BE cells compared to MCF7 cells has been identified, by computational analysis, as a 2.5 times slower rate of p53 degradation [221]. This slower rate of degradation in BE cells is accompanied by an increase in p53 expression noise (0.55), while MCF7 cells when treated with Nutlin, an MDM2 inhibitor, accumulate higher levels of p53 which displays alteration in p53 expression noise to (0.77) [222]. The measured increase in expression noise as protein copy numbers increase over $\sim$ two orders

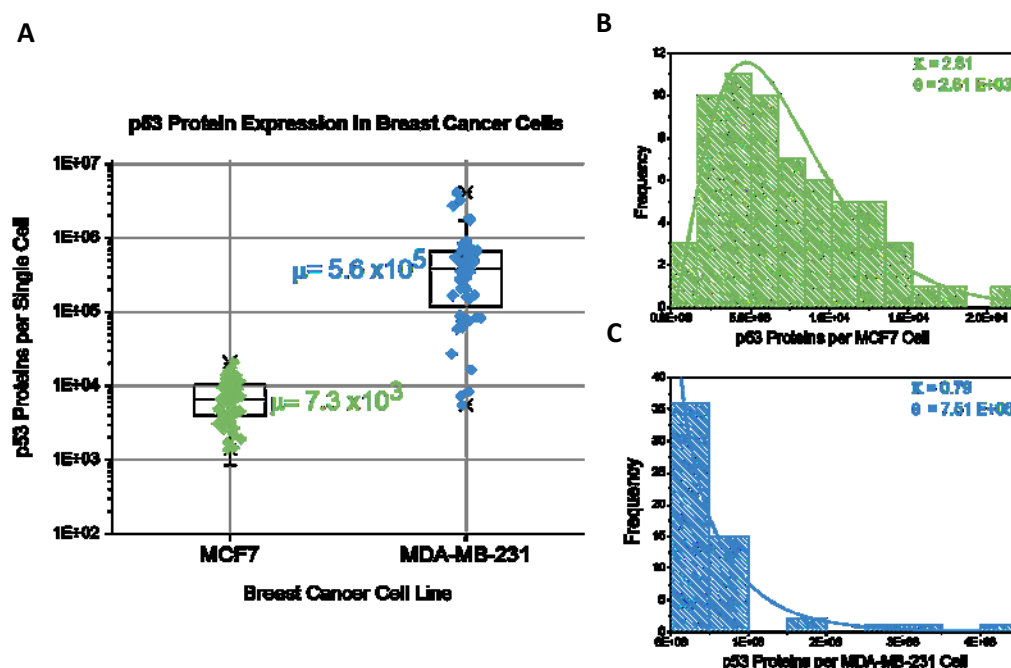


Figure 5.18 Expression of p53 in MCF7 & MDA-MB-231. (A) The 1.75nL Flip Chip B measured wildtype p53 expression in an MCF7 cell is 7.3×10^3 . While the MDA-MB-231 cell has an average expression level of 5.6×10^5 this is 76 times more p53 compared to the MCF7 cell. Single cell distributions of p53 expression fit with a gamma probability distribution for (B) MCF7 and (C) MDA-MB-231 breast cancer cells. The shape parameter (K) for the distribution decreases from 2.81 to 0.79, respectively.

of magnitude provides disagreement with, previously discussed in section 1.5.2 the scaling behaviour for gene expression in yeast, E. coli, and mammalian cells. However, an explanation for this increase in noise above the extrinsic noise limit may be altered protein expression under stress perturbed conditions [88]. To further understand the dynamics of noise creation within these p53 distributions a separate chapter, titled *Sources of Noise in Gene Expression* should be referred. This Chapter 7 further explores know variables associated with the central dogma of molecular biology and the impact on p53 distributional shape parameters.

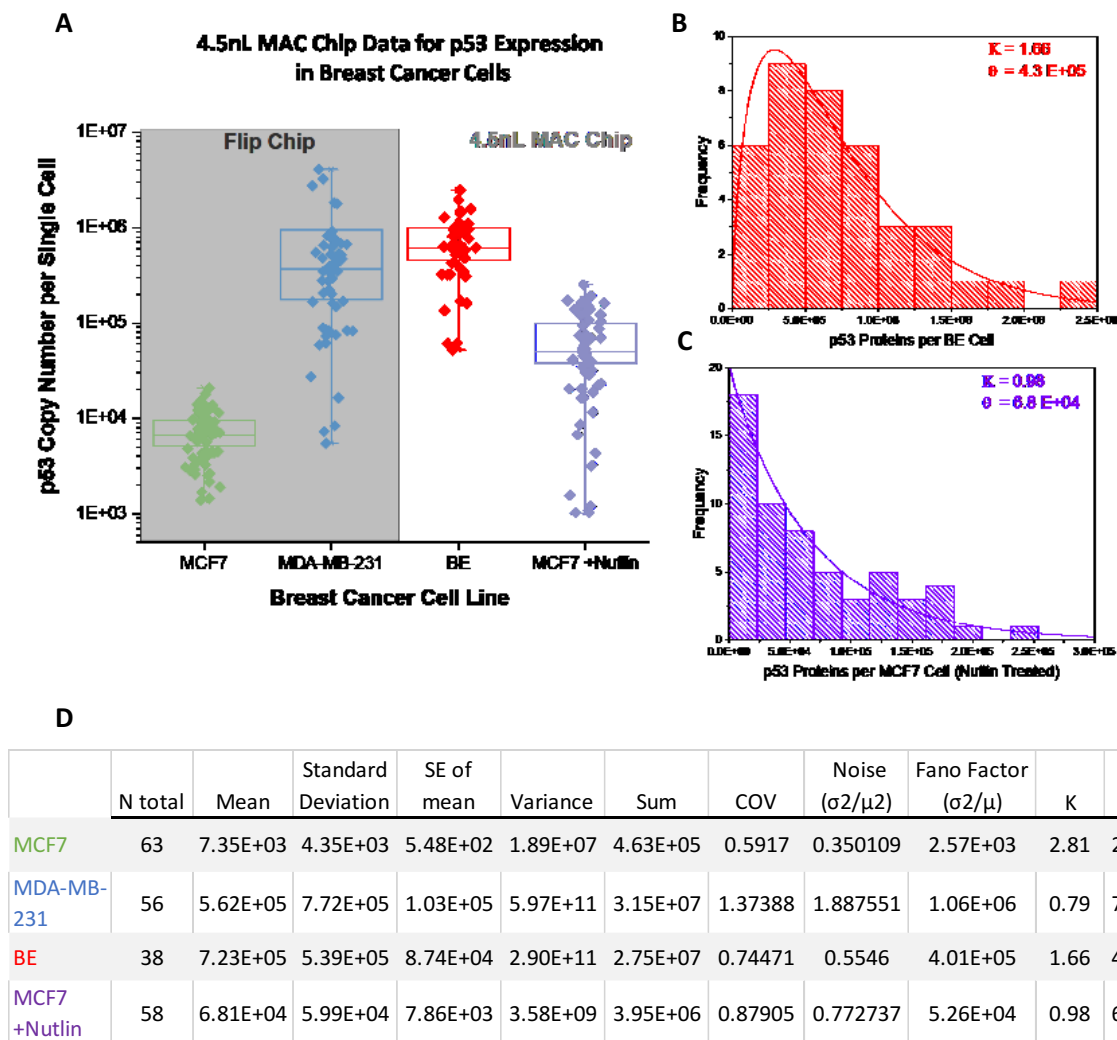


Figure 5.19 Comparison with 4.5nL p53 Expression MAC Chip Measured Cell Lines. (A) Increasing our experimental measurements upon p53 content by including two previously measured cell lines, it can be shown that a wide variability of p53 expression noise exists. (B) Modulation of p53 degradation rates either naturally occurring in BE cells or (C) pharmacologically altered by the addition of Nutlin an increase in the gamma distributional tail is measured. Single molecule MAC Chip experiments for p53 content were performed by Vanessa Ho and Dr. James Squires respectively. (D) A descriptive statistical analysis of p53 expression has also been included.

5.6 Conclusion

Developing cell trapping structures arranged in a microarray format, a microfluidic device titled the Flip Chip has been fabricated. Two design approaches were originally taken, one design, Flip Chip A, consisted of a two-chamber structure. This design consisted of a deep $\sim 20\ \mu\text{m}$ cell trapping chamber which resided inside a larger diameter antibody alignment chamber which was $\sim 7\ \mu\text{m}$ in depth. Due to alignment complications of both the photolithography processes and antibody spot alignment with the two-chamber structure, this was only produced as a microarray of 25 chambers. The other design approach was Flip Chip B, single chambers were fabricated with a depth of $25\ \mu\text{m}$ and a diameter of $150\ \mu\text{m}$, this was created as a microarray of 300 chambers. When this chamber was placed upon the coverslip which had been surface functionalized with a capture antibody the diameter of the chamber correlates with the size of the capture antibody spot per the chamber.

Flip Chip A was implemented in preliminary experiments by using a previously developed p53 assay with BE cells to validate the operational procedure. From this experiment, a positive correlation is presented between the number of BE cells within a chamber and the measured antibody spot fluorescence, presented in *Figure 5.9*. Following from this experiment Flip Chip B was implemented in absolute quantification of p53 expression from single breast cancer cells after calibration experiments for the p53 assay were performed. The wild-type p53 expressing cell line showed an average expression of $7.3 \times 10^3 \pm 547$ p53 molecules. While the overexpressing p53 cell line expressed on average $5.6 \times 10^5 \pm 1.03 \times 10^5$ p53 molecules, shown in *Figure 5.19*.

The advantages of implementing the Flip Chip have been demonstrated initially through comparison of p53 assay calibration curves. Presenting that a reduction in chamber volume and an increase in antibody capture spot diameter provides a higher concentration of antibody, in return this provides a higher antigen capture percentage. This increase has allowed the Flip Chip 1.75 nL chamber to display an antigen capture percentage comparable to that of a 0.75 nL MAC Chip, seen in *Figure 5.16*. The second advantage is presented in the operational workflow procedure, where the loading of single cells into chambers can be accomplished in under ten minutes. While loading cells through optical trapping in the MAC chip may take users over thirty minutes. However, a disadvantage to the Flip Chip operational protocol is the limitation in single cell capture. As increasing the concentration of cells to increase cell capture events leads to a disadvantageous increase in the before assay background binding events. This limitation allows the user of the device to trap cells quicker, yet the throughput of single cell analysis is similar to that of one MAC chip experiment.

Chapter 6

Quantitative Fluorescence Microscopy

To expand the convenience of quantitative proteomics, this experimental chapter explores a synergistic approach for fluorophore quantification in three fluorescent cell lines by employing both epi-fluorescence microscopy and the microfluidic affinity capture chip. The concordance between the measurement techniques allows the ratiometric calibration of a fluorescent secondary standard. This standard is then implemented in a proof of concept experiment permitting quantitative epi-fluorescence microscopy for the fluorescent protein content in chloroplasts.

6.1 Introduction

Genetically encoded fluorescent proteins (FPs) and fluorescent protein fusions (FPFs) have become powerful tools in plant molecular biology research. FPFs have allowed breakthroughs in spatial and temporal relative measurements of protein expression patterns while FPs provide information towards cell-type specific transcriptomics and demarcating cell fate identity during root or leaf development. FPs have also proven validity as biological sensors, where the expression of an FP is driven by the presence or absence of a specific signal, such as hormones or the presence of minerals [223].

While the exploitation of FPs and FPFs continue to develop and grow, so has the appreciation to evolve the qualitative data (arbitrary units of fluorescence intensity) towards quantitative measurements [224]. This provides protein copy numbers allowing rates of biological processes to be determined and values for mathematical modelling with higher accuracy [225]. Through super-resolution microscopy techniques, single molecules can be counted within live cells by either directly measuring the intensity of a single GFP molecule or measuring a concentration range of GFP molecules, allowing this qualitative data to be calibrated towards quantitative. This technique of calibration was recently applied to quantify the Brassinosteroid Insensitive1 (BRI1) receptor in Arabidopsis root cells [226]. Furthermore, another powerful method to quantify protein concentration was by inserting a protein quantitation reporter into the genome between the GFP sequence and the gene of

interest. After transcription and translation, the result is the production of GFP at a 1:1 ratio with the protein of interest, providing relative measurements of protein concentrations [227].

Fluorescent reporter techniques have presented an alternative method to overcome the inaccuracies and variability witnessed for *in planta* immunodetection quantitation. These negative consequences are the product of the antibodies used lacking the necessary avidity or affinity, a failure to access the antigen binding sequence due to in-vivo modifications such as phosphorylation, and limited detection capabilities due to the cross-reactivity of chosen antibodies. Yet, FPs and FPFs still contain obstacles for transgenic plants, one of these setbacks was recognised early in *Arabidopsis* as a failure to produce stable GFP expressing transformants. It was later identified that through aberrant mRNA processing a segment of the GFP codon was spliced due to sequence similarity to those of normal plant introns. Proper GFP expression in transformants was restored by mutation of the recognised GFP codon [228]. Other drawbacks specific to FPFs are tagging-induced artefacts, that is the addition of a fluorescent tag upon a protein for detection producing aberrant results for *in planta* localisation. This can be caused by misfolding or misassembling of fusion proteins causing entrapment within the endoplasmic reticulum, failure to traffic through organelles for protein sorting, and endomembrane compartments restricting the movement of FPs [229].

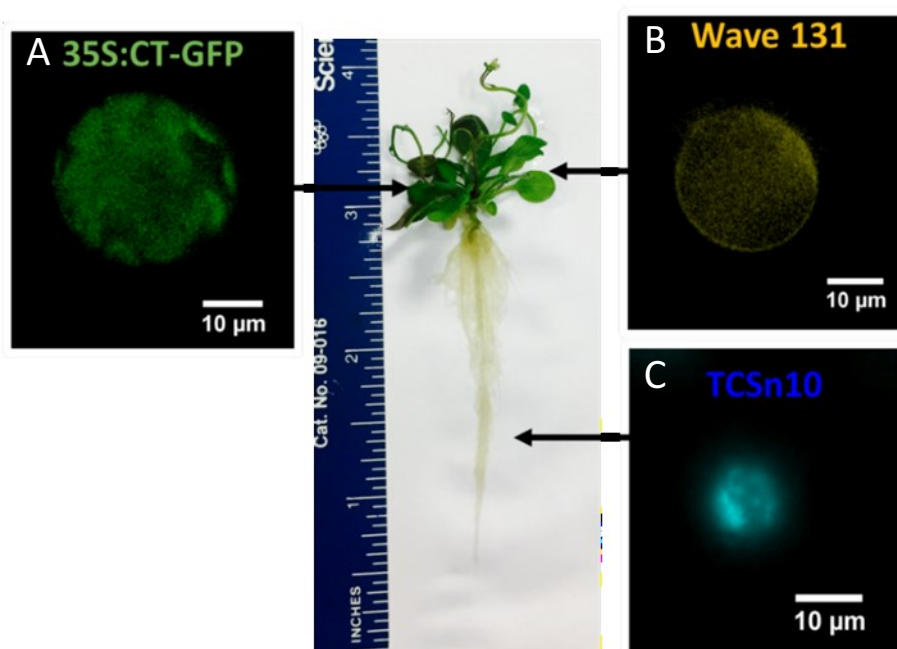


Figure 6.1 Three Transgenic Arabidopsis Cell Lines Implemented for Quantitative Epi-Fluorescence. Epi-Fluorescence images from the three cell lines chosen, single cells were isolated from either leaf or root sections. The first two cell lines, (A) 35S: CT-GFP and (B) Wave 131 were isolated from leaf sections, fluorescent proteins were found in the chloroplast stroma or cellular membrane respectively. (C) Protoplasts from the TCSn10 cell line were isolated from root sections, as can be seen the fluorescent proteins were randomly dispersed within a root protoplast.

However, with these caveats, the advantages of FPFs and FPs over traditional methods are superior in generation, detection, and provide the opportunity to study protein expression, transcriptomics and even the complete signalling pathway in live cells [39,185]. This has been previously demonstrated in single *E. coli* and *S. cerevisiae* cells for global profiling of protein copy number and expression noise through the employment of FPFs [89,90]. The three cell lines measured in this chapter, presented in *Figure 6.1*, exemplify the variation of FPs and FPFs that are widely available from seed banks. One cell line, the 35S:CT-GFP, is a native FP which is targeted to the chloroplast by the addition of a transit peptide sequence [154]. The second cell line is Wave 131 an FPFs, allowing both locational and quantitative information [230]. While the final cell line measured is TCSn:10, showing the ability of an FP to be utilised as a biosensor towards auxin stimulation [231].

Advances in the acceptance and methodology for implementation of FPs and FPFs have been made possible by parallel improvements in the image acquisition ability of fluorescent microscopy. Such as cameras with greater than 90% peak quantum efficiency with very low read noise. Various fluorescent microscopy techniques are available, such as confocal microscopy, multiphoton microscopy, light-sheet microscopy, and epi-fluorescence microscopy [228]. All microscopy techniques provide various advantages and disadvantages concerning fluorophore bleaching rates, image resolution, and expense. Considering these techniques, we have opted for epi-fluorescence microscopy for providing measurements upon these three transgenic cell lines as it was readily available and the cheapest, most straightforward, fluorescence microscopy technique on the market.

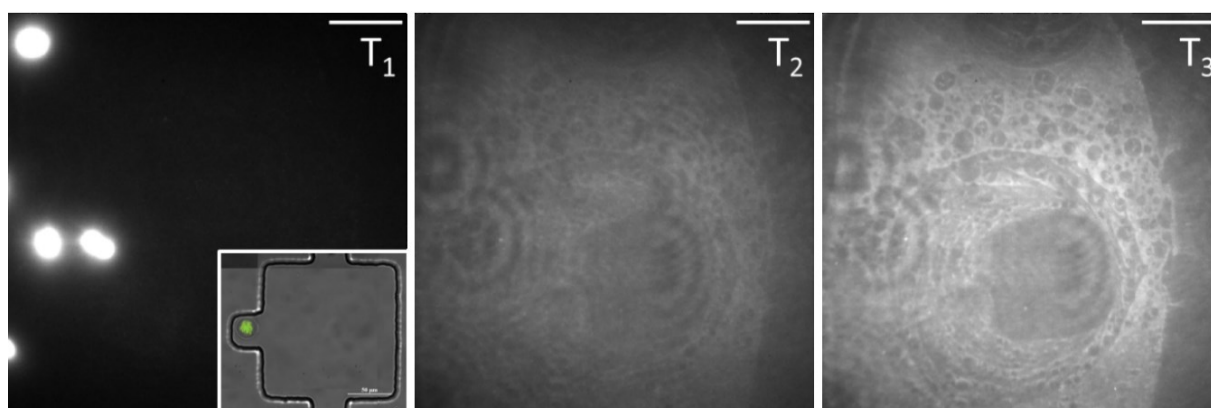


Figure 6.2 MAC Chip Raw Experiment Images for a 35S: CT-GFP Mesophyll Protoplast. Single mesophyll protoplasts were isolated into 0.75nL MAC chip assay chambers, as shown (inset of T₁). (T₁) Upon optical lysis the chloroplasts, containing the GFP, were liberated into the assay chamber. After the induction of lysis buffer, the GFP is released and binds to the antibody spot printed within the assay chamber fabricating the completed bimolecular detection assay, (T₂) shown at 15 minutes and (T₃) 45.

6.2 Additional Materials and Methods

Additional materials and methods have been applied to explore the ability to increase the throughput of the MAC chip methodology by incorporating epi-fluorescence microscopy measurements. The three chosen transgenic cell lines, previously presented in *Figure 6.1*, undergo absolute fluorescent protein quantification by the MAC Chip methodology, specifically through two experiments, allowing the average quantification of 60 cells. This is followed by the application of epi-fluorescence microscopy to measure a 2.5-fold larger data set of 155 cells.

6.2.1 Plant Material

Mesophyll cells were isolated from the transgenic cell lines Wave line 131 and 35S: CT-GFP following the protocols outlined in section 2.6.1 under soil growth conditions, and the mesophyll protoplast isolation was described in section 2.6.2. Root cells were isolated from the transgenic cell line TCSn:10 which undergo growth in a sterile environment, outlined in section 2.6.1, while isolation was performed following the methods detailed in section 2.6.3.

6.2.2 Measurements by Microfluidic Affinity Capture Chip

Absolute quantification of fluorescent proteins through the MAC Chip workflow has previously been outlined in section 2.8, specifically in *Figure 2.16*. The capture antibody applied is the same as implemented in section 2.8.3, an anti-GFP monoclonal antibody raised in mice and donated by Professor P. Parker (Cancer Research UK, Lincoln's Inn Fields Laboratories). The antibody, after dialysis in PBS, was diluted with Array-it protein printing buffer to a final concentration of 0.45 mg mL^{-1} and was printed following the protocols outlined in section 2.3, specifically the microarray fabrication detailed in section 2.3.3. Quantification of fluorescent protein binding events upon this antibody capture spot follow the methods outlined in section 2.4. Raw experimental image from a MAC chip experiment can be seen in *Figure 6.2*.

6.2.3 Measurements by Epi-Fluorescent Microscopy

The experimental platform for arbitrary fluorescence intensity quantification by widefield microscopy has been detailed in section 2.4.1. The theory of quantification by epi-fluorescence microscopy has been described in section 2.4.4. Accompanied by a visual depiction discerning the measurements of fluorophore concentration or total fluorophore amount which has been provided in *Figure 2.9*. Fluorescence intensity measurements were

achieved through pipetting a 30 μ L solution of transgenic protoplasts in W5 4% BSA upon a poly-lysine coated coverslip. Followed by cell sedimentation for 15 minutes then imaging.

Preliminary experiments, presented in *Figure 6.3*, were performed to empirically assess the appropriate method of two-dimensional or three-dimensional image acquisition for GFP measurements within a single cell. While 3D Epi-Fluorescence measurements are necessary for accurately determining an intensity of an object larger than the diffraction limit, draw-backs for this method exist in the form of photobleaching or the time necessary for image acquisition. While 2D fluorescence acquisition from the in-focus image plane of specimens with a low-level of out-of-focus fluorescence, similar to that of adherent cell cultures, may result in the highest signal to noise ratio [36,37].

The fluorescence intensity per protoplast was imaged through a 60X oil-immersion objective (NA=1.49) in a well-focused 2D image or a 3D z-stack image acquired by 1 μ m slices over 40 μ m. Excitation of the GFP fluorophore was performed through a mercury lamp (Nikon, Japan) and a Nikon standard FITC filter set (FITC-2024B-NTE; Nikon, Japan). The camera was set for 1x1 binning with an exposure of 200 ms with a photon multiplier and conversion gain of 10 and 2.4x, respectively. The 3D images underwent deconvolution using a point spread function for GFP, generated in “DeconvolutionLab2” a software package created by the biomedical imaging group at EPFL. Deconvolution allows the elimination of fluorescence distortion produced by out of z-plane fluorescence signals [232]. The 3D images were quantified by summing the fluorescence intensity generated in each z-plane for a single cell. This was compared, as shown in *Figure 6.3 C*, to that of the 2D in-focus image acquired for the same cell after background subtraction, this process is described by *Equation 6.2*. A high correlation between the two measurements $R^2 = 0.94$ and this linear agreement was shown for over four orders of magnitude. Providing evidence that for a single cell a well-focused 2D image which has undergone background subtraction can provide a similar distribution to that of a 3D image.

Concerning this agreement, the implementation of well focused-2D images obtained through epi-fluorescence microscopy was chosen, and the images can be described by *Equation 6.1*. Where a fluorescent image ($P(x,y)$) is a product of illumination ($I(x,y)$), distribution of detection efficiency ($D(x,y)$), fluorescent distribution from pixel to pixel over the specimen ($F(x,y)$), and the image exposure time ($T(s)$) [233].

$$P(x, y) = I(x, y) \cdot D(x, y) \cdot F(x, y) \cdot T(s) \quad (6.1)$$

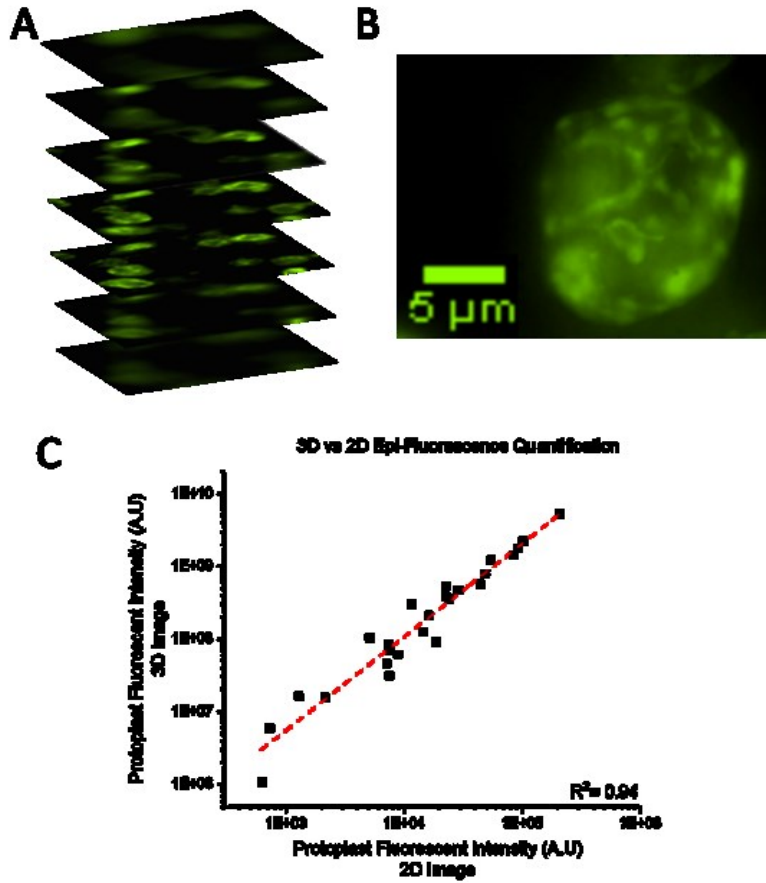


Figure 6.3 Fluorescence Intensity Quantification 2D vs 3D Image Acquisition. Signal for GFP fluorophore content within root protoplasts was defined as the integrated intensity per cell acquisitioned by either (A) 3D or (B) 2D image analysis. (C) Comparing the integrated intensity of both techniques for the same cell presents a high level of correlation between measurements ($R^2=0.94$) for over four orders of magnitude. Providing evidence that 2D images will present similar fluorophore content per cell distributions to those acquired by 3D images.

As previously discussed in section 1.3.5 the accuracy of quantitation through fluorescence microscopy is dependent upon the subtraction of the background fluorescence [37]. Measuring this background fluorescence value ($P_{bkg}(x,y)$) is achieved following the previously discussed image acquisition parameters on a solution of supernatant from the corresponding isolated transgenic protoplasts. The background fluorescence signal contains an identical number of pixels and is subtracted from the fluorescence image ($P(x,y)$) following from *Equation 6.2*, where F is the fluorescence intensity measured at each pixel i (pixel in the object) or j (pixel in the background), and obj or bkg denotes object of interest or background respectively. N , the number of pixels in the object or background measurement are equal values of observations. An example of this procedure can be seen in *Figure 6.4*.

$$F_{obj} = \sum_{i=1}^{i=N_{obj}} F_{obji} - N_{obj} \frac{\sum_{j=1}^{j=N_{bkg}} F_{bkgj}}{N_{bkg}} \quad (6.2)$$

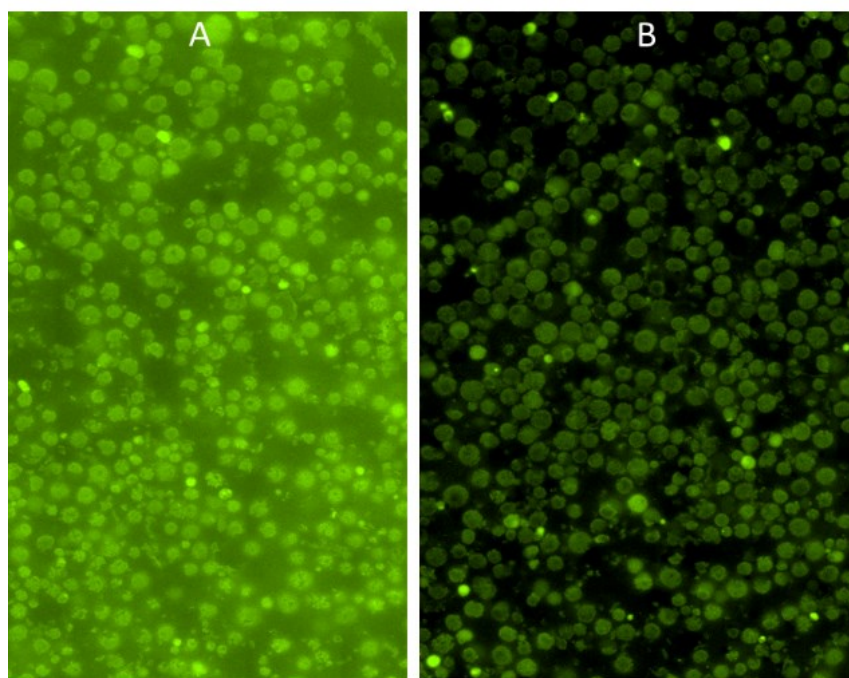


Figure 6.4 Large 60x Tile Scan Epi-Fluorescence Measurements. (A) Epi-fluorescence images were acquired after the sedimentation of a 30 μ L solution of mesophyll protoplasts upon a poly-lysine coated coverslip. Image acquisition is through implementing a 60x NA=1.49 oil immersion objective in tile scan mode, where the z-axis movement is under control of a perfect focus system. (B) The resulting images undergo background subtraction from culture solution supernatant, revealing the true signal of GFP expression within the protoplasts (B).

6.3 Green Fluorescent Protein Calibration Curve

To be able to determine the absolute GFP content within a single cell a calibration curve must first be obtained, where a known amount of GFP was spiked into a W5 4% BSA solution ranging from 10^3 to 10^8 GFP molecules per nL and introduced into the MAC chip analysis chambers. This allowed the ability to turn the digital readout of GFP molecules bound to the antibody spot into a quantitative number respective of the amount of GFP molecules present within the analysis chamber.

6.3.1 Fluorescent Intensity of Fluorescent Proteins

To perform a quantitative analysis of GFP binding events in the congested regime, measurements of the single molecule fluorescent intensity for GFP is necessary. This is achieved by introducing 10^3 GFP molecules per nL into analysis chambers, which do not contain a capture antibody spot. Images were acquired similar to that of *Figure 6.5 A*. Through the implementation of the single molecule localisation program, discussed in section 2.4.3, single GFP fluorophores were identified as presented in *Figure 6.5 B* and quantified for single molecule intensity. The average single-molecule intensity of a GFP fluorophore was 1.9 as displayed in *Figure 6.5 C*. Using this mean intensity measurement congested antibody spots, after laser profile intensity subtraction, can be quantified for GFP binding events.

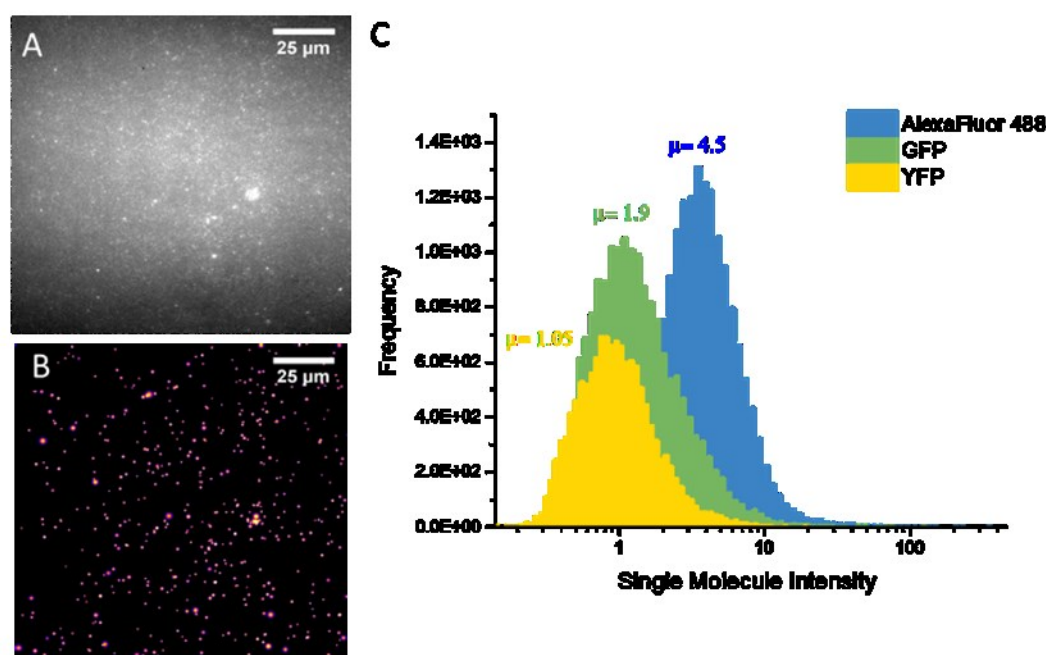


Figure 6.5 Fluorophore Fluorescence Intensity. Single molecule intensity measurements are performed from (A) introducing spatially separated fluorophores on a coverslip followed by (B) fluorophore recognition through single molecule localization algorithms. (C) Application of this method allows the quantifiable average of the fluorescence intensity (quantum yield) of a fluorophore (GFP and YFP) or multiple fluorophores bound to an antibody (AlexaFluor 488). This measurement is implemented in congested image analysis, previously discussed in section 2.4.3, to determine the number of fluorophores in an image.

6.3.2 Calibration Curve for GFP Bi-Molecular Assay

As previously shown in the device validation experiments, in section 2.8.3, the GFP capture efficiency of the antibody assay is far superior, by 52%, in 0.75 nL analysis chambers compared to that of 4.5 nL chambers. Therefore, all MAC chip experiments and calibration experiments for this chapter were performed in 0.75 nL analysis chambers. To produce a calibration-curve a known amount of GFP was spiked into a W5 4% BSA solution and introduced into the 0.75 nL MAC chip analysis chambers. Images of antibody spots at assay equilibrium were quantified for the number of GFP binding events. This calibration allows the number of molecules bound to the capture antibody spot to be a measurement of the absolute amount of GFP molecules in the analysis chamber.

The calibration curve for the GFP assay is shown in *Figure 6.6 A*. It was shown that on average 10% of the GFP molecules in the analysis chamber were captured and quantified upon the antibody spot. However, the assay does approach a level of antigen binding saturation when 7.5×10^7 GFP molecules were in the analysis chamber. This is demonstrated by the decline of available GFP binding antibodies and only capturing 5% of the available GFP producing a decreased linear response within the calibration curve. The curve can be fit to a Hill function which demonstrates the sigmoidal curve of analyte binding events upon a

macromolecule with respect to a change in analyte concentration. The Hill coefficient for this assay is $n = 0.92$, showing independent binding concerning the concentration of analyte molecules. The linear regime of the assay, which was used to translate single cell GFP binding events to absolute GFP content per single cell, is the region described by a linear fit of ($y = 0.051x$).

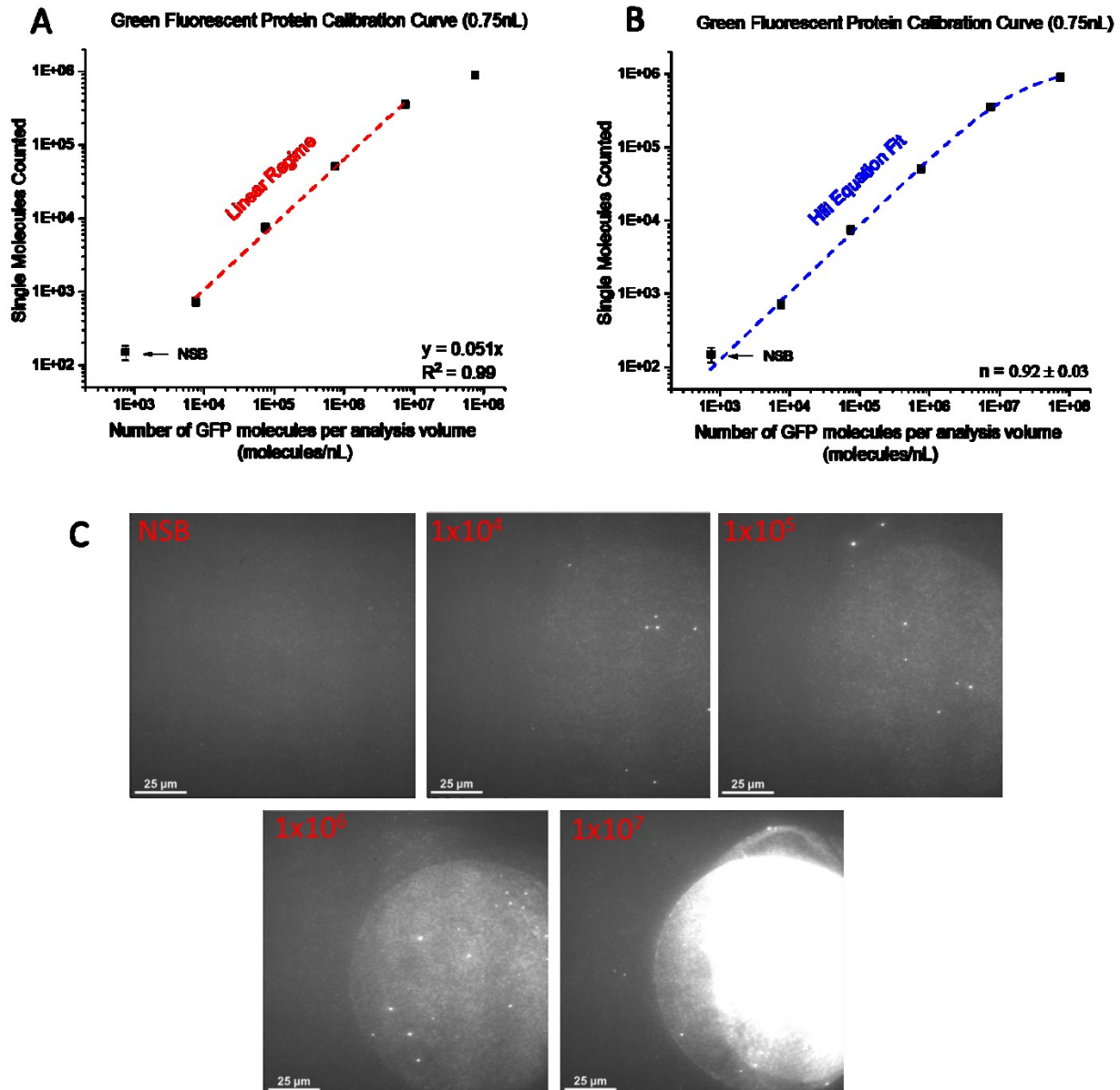


Figure 6.6 GFP Calibration Curve for 0.75 nL Assay Chambers. A known amount of GFP is introduced into 0.75 nL microfluidic assay chambers. (A) After equilibrium has been reached ($t=60$ minutes) the bound GFP is identified by single molecule binding events quantified by fluorophore intensity measurements presented in Figure 6.5. A linear fit of ($y=0.051x$) is identified between the GFP (molecules/nL) introduced into the assay and the binding events measured (single molecule count). The linear range allows a standardized GFP content to be recognized with only the identification of the single molecules bound at assay equilibrium. (B) To assess the relationship of the bi-molecular assay a Hill function fit to the calibration curve. Presenting a coefficient of 0.92, which identifies that the affinity of the capture antibody spot for the antigen (GFP) does not alter as the number of GFP binding events increase. (C) Raw images of GFP binding events in the linear range of the 0.75 nL chamber GFP calibration experiment, where the above number denotes the GFP molecules/nL present in the chamber, while NSB (non-specific background) is the assay measured with no GFP present. The LUTs have been scaled identical in each image to allow the identification of GFP binding events when 1×10^4 GFP molecules/nL are present.

6.4 35S: CT-GFP Cell Line

The first quantified transgenic *Arabidopsis thaliana* cell line was 35S: CT-GFP, where the cauliflower mosaic virus drives the nuclear expression of GFP which is targeted to the chloroplast stroma [154].

6.4.1 Cauliflower Mosaic Virus Expression of GFP

The cauliflower mosaic virus is a plant pararetrovirus, containing DNA rather than RNA genomic material to introduce infection into the plant host. The host's polymerase readily transcribes this virus's genomic material as it recognises the 35S promoter, this creates devastating necrotic lesions on leaf surfaces and deformation of overall plant development [149]. Franck et al. first discovered the nucleotide sequence of the cauliflower mosaic virus, in the 1980's, and at this moment in time, the 35S promoter is one of the most commonly used promoters in plant biotechnology.

The 35S promoter is constitutively active. Therefore there is a continual transcription of the gene leading to a high and variable amount of protein expression. This high level of expression is witnessed in dicots while overexpression has been shown to be less effective in monocots [234]. *Arabidopsis* is a dicot, therefore the ideal species for this method of transgenic modification. The 35S: CT-GFP cell line contains nuclear DNA that has been genetically modified so that a GFP coding region is connected to the transit peptide from the nuclear-located chloroplast *recA* gene. This entire coding region is under the expression control of the 35S promoter, allowing a high level of GFP to be targeted into the chloroplast stroma.

6.4.2 MAC Chip Measurements

To determine the absolute copy number of GFP within single mesophyll protoplasts, the previously discussed methodology in section 2.8 has been applied. Where single mesophyll protoplasts were isolated from healthy 35S: CT-GFP *Arabidopsis* leaves, introduced into analysis chambers within the MAC chip and analysed for GFP content using the bi-molecular capture assay. The application of this bi-molecular assay within MAC Chip assay chambers was previously displayed in *Figure 6.2*. The capture assay consists of Array-it protein printing buffer with an anti-GFP antibody at a final concentration of 0.45 mg mL⁻¹.

The amount of GFP molecules bound to each antibody spot per protoplast on average was $2.05 \times 10^5 \pm 2.55 \times 10^4$, this was the highest fluorophore producing cell line of the three analysed. These binding events were translated, through the application of the previously

obtained calibration curve, into absolute GFP content per single cell with an average GFP copy number of $4.5 \times 10^6 \pm 5.4 \times 10^5$. This protein copy number distribution exhibits a gamma distribution with a K parameter of 1.59 which mimics that previously seen in section 5.5 concerning proteins under non-wild-type regulation. It is expected and previously shown that the expression distribution of a protein under a strong constitutive promoter would be classified under noisy expression, where the protein expression noise is classified as (σ^2/μ^2) , or the coefficient of variation squared [235]. The protein expression noise for GFP in the 35S: CT-GFP cell line is 0.63 which is a 6- fold increase than the previously proposed extrinsic noise floor of ~ 0.1 [85].

6.4.3 Epi-Fluorescent Measurements

To turn epi-fluorescent microscopy into quantitative numbers rather than arbitrary intensity units, 35S: CT-GFP protoplasts were quantified for fluorescence intensity through epi-fluorescent microscopy following the methods outlined in section 6.2.3. The average intensity

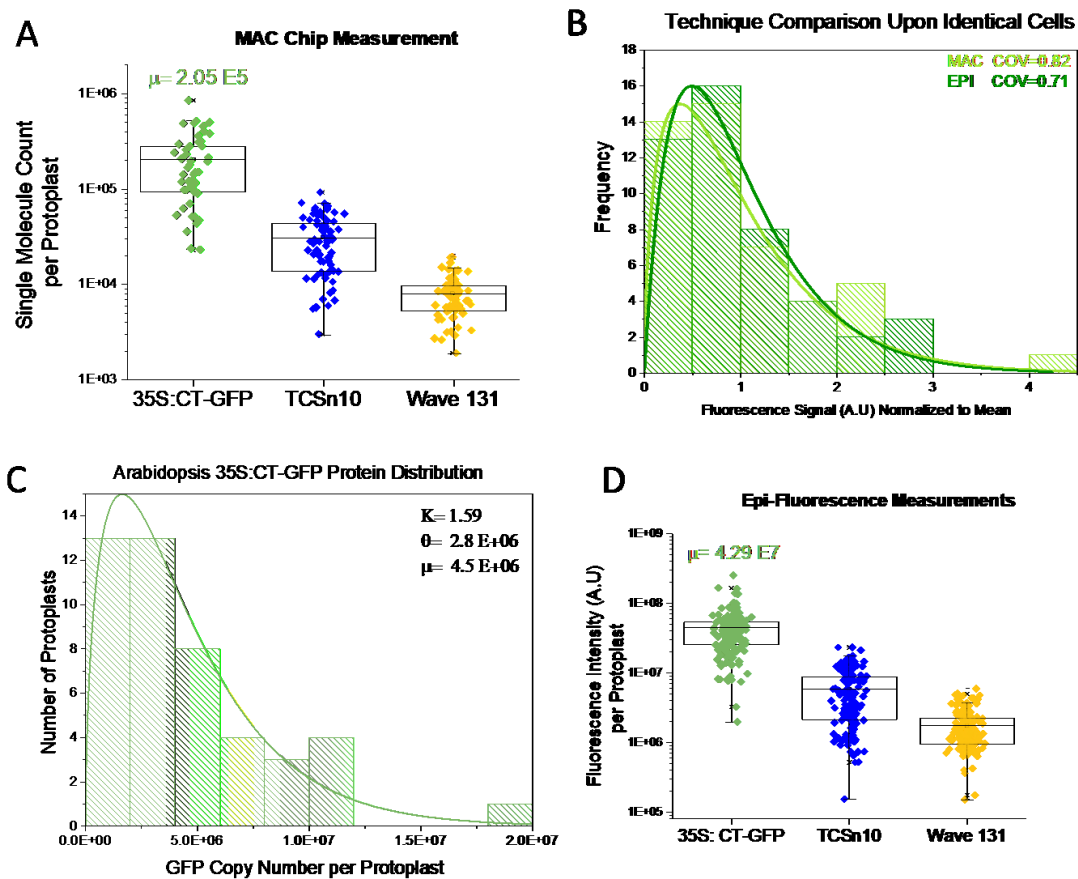


Figure 6.7 Compiled Measurements on 35S: CT-GFP Protoplasts. The 35S: CT-GFP cell line expressed the highest content of fluorophores per cell, (A) with MAC Chip measurements quantifying 2.05×10^5 GFP binding events. (B) Summing the GFP intensity of before lysis epi-fluorescence images and comparison to single molecule binding events in the MAC chip for the same cell presents an 89% similarity between the COV for the two distributions. (C) Implementing the calibration curve of GFP binding events the absolute copy number of GFP per protoplasts is calculated to be 4.5×10^6 . (D) While the high-throughput epi-fluorescence measurements present an average fluorescence intensity of 4.29×10^7 .

of a 35S: CT-GFP cell is $4.29 \times 10^7 \pm 2.8 \times 10^6$ as shown in *Figure 6.7 D*. With comparison to the other cell lines analysed the 35S: CT-GFP presented the highest average fluorescent intensity per protoplast. The epi-fluorescent data is composed of 157 35S: CT-GFP cells and the image acquisition was under 30 minutes. Presenting a 3-fold increase in cells quantified and a 16-fold reduction in time spent in the lab with a comparison to the 50 cells analysed by performing two MAC chip experiments, taking an average of 4 hours per experiment, demonstrating a movement toward an increasing throughput through the implementation of epi-fluorescent microscopy measurements.

6.5 Wave 131 Cell Line

The second quantified transgenic *Arabidopsis thaliana* cell line was Wave 131. This cell line has been modified to contain an FPFs, where Novel plant SNARE12 (NPSN12), a membrane protein, is fused to YFP. Demonstrating the ability to utilise a fluorophore as a quantitative measurement tool as a 1:1 stoichiometric ratio of protein produced. Further advantages such as protein localisation can be witnessed through implementing epi-fluorescent measurement techniques.

6.5.1 SNARE12: YFP Fusion Protein

The “wave cell line” is a collection of multicolour FPFs and allows the visualisation of membrane compartment markers allowing further understanding of plant membrane trafficking. These cell lines have been thoroughly investigated for consistent expression patterns, the agreement of localisation with previous literature reports, and an ability to produce identical expression within progeny [230]. We have applied one of these cell lines titled Wave 131 as an opportunity for the first time to quantify a membrane located protein, visually apparent in *Figure 6.8*. While also demonstrating the value of FPFs within the MAC chip absolute quantification methodology.

NPSN12 has been shown to localise within the plasma membrane. Through reverse transcriptase-PCR analysis the homogeneous expression of RT-PCR product for NPSN12 have been found throughout all cells in planta [236]. The molecular function of this protein remains unknown, yet speculations can be made based upon sequence similarity showing functionality as a vesicle trafficking protein within the secretory pathway [237].

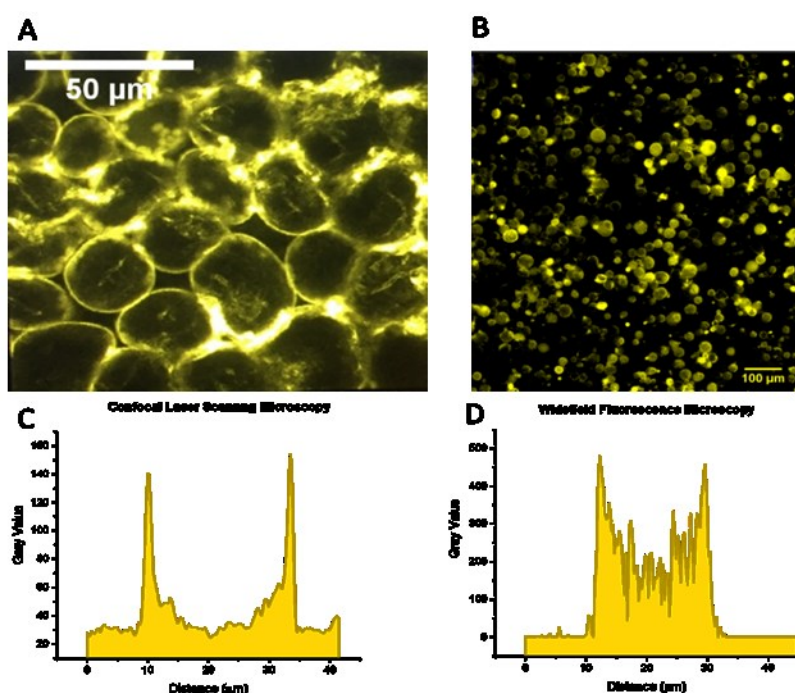


Figure 6.8 Intra-Cellular Localization of NPSN12. Image acquisition by confocal laser scanning microscopy (CLSM) within (A) intact live leaf tissue and (B) widefield epi-fluorescence microscopy of isolated protoplasts identify the single cell localization of fluorescent fusion protein NPSN12. Single cell fluorescence intensity profiles of (C) CLSM and (D) widefield fluorescence microscopy demonstrate a higher fluorophore intensity within the membrane region compared to the cytosolic region.

6.5.2 MAC Chip Measurements

The same methodology, outlined in section 6.2.2, for MAC chip GFP measurements, was applied to determine the absolute copy number of YFP within Wave 131 mesophyll protoplasts, as the bimolecular GFP antibody assay maintains the same monoclonal antibody affinity for YFP as GFP due to the 97% overall sequence similarity. The previously calculated GFP calibration curve was used, yet binding events were measured by the average single-molecule intensity of a YFP fluorophore measured to be 1.05. The average amount of YFP bound to the antibody spot after cell lysis was $8.53 \times 10^3 \pm 530$, the lowest fluorophore expressing cell line of the three. Translating to an absolute copy number of YFP per protoplasts being $1.7 \times 10^5 \pm 1 \times 10^4$. As there is a 1:1 stoichiometric ratio concerning the composure of this FPF, providing evidence that NPSN12 absolute copy number would also be $1.7 \times 10^5 \pm 1 \times 10^4$.

As the expression of the FPF in this cell line is under wild-type control, the noise witnessed in NPSN12 expression would be expected to be lower than the non-wildtype expression of GFP under the 35S promoter. Due to the measured copy number of 1.7×10^5 , one would expect the noise to be similar to the wild-type MCF7 p53 expression noise of 0.329. The measured noise for this cell line was 0.36

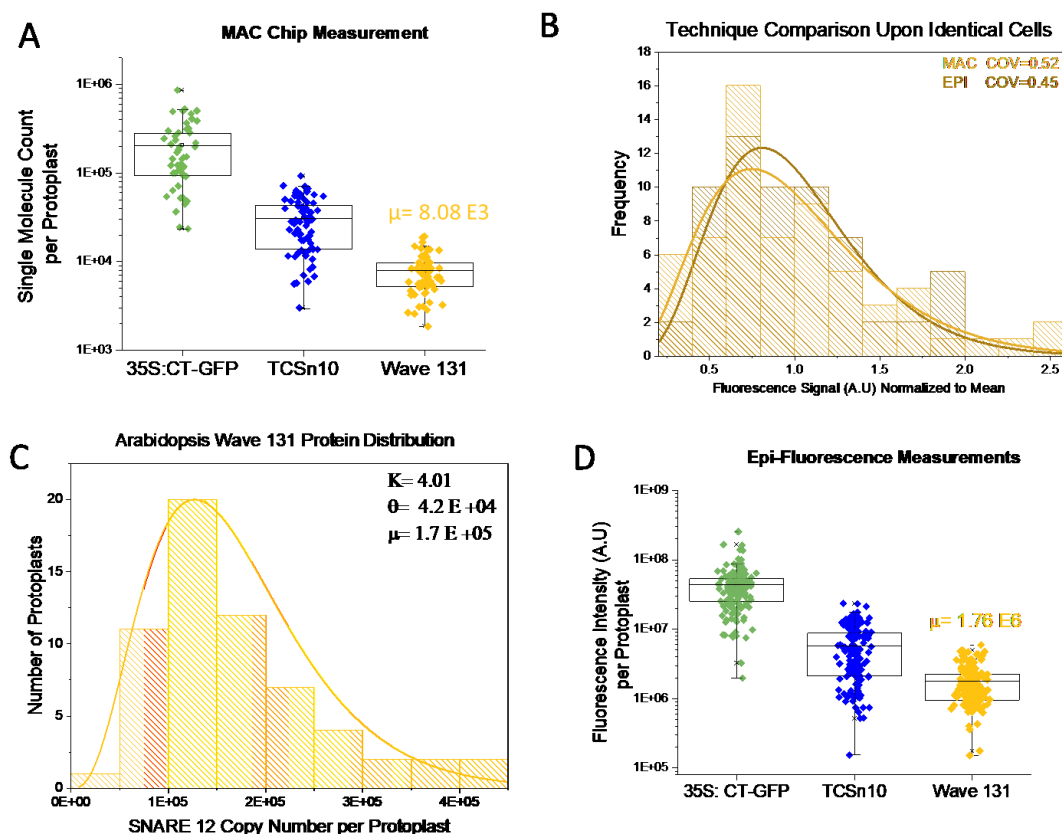


Figure 6.9 Compiled Measurements on Wave131 Protoplasts. The Wave 131 cell line expressed on average the lowest fluorophore content per cell, with (A) MAC Chip measurements quantifying 8.53×10^3 YFP binding events. (B) Summating YFP intensity for the before lysis epi-fluorescence images and their comparison to single molecule binding events in the MAC chip for the same cell presents an 86% similarity between the COV for the two distributions. (C) Implementing the calibration curve of GFP binding events the absolute copy number of YFP per protoplast is calculated on average to be 1.7×10^5 . (D) While the high-throughput epi-fluorescence measurements present an average fluorescence intensity of 1.76×10^6 .

6.5.3 Epi-Fluorescence Measurements

Wave 131 mesophyll protoplasts were isolated and imaged upon a coverslip using the previously used parameters for image capture and background subtraction, described in section 6.2.3. The average intensity for the protoplasts was $1.76 \times 10^6 \pm 2 \times 10^5$. The epi-fluorescence images gathered for this cell line presents localisation of NPSN12 to be within the plasma membrane as shown in *Figure 6.8*.

6.6 TCSn10 Cell Line

The third quantified transgenic *Arabidopsis thaliana* cell line was TCSn10. This cell line has been modified by the incorporation of a GFP sensor into the genome to monitor the transcriptional activity of type-B *Arabidopsis* response regulators (ARR). Demonstrating the ability to quantify the cell to cell differences in a proteins transcriptional activity [91].

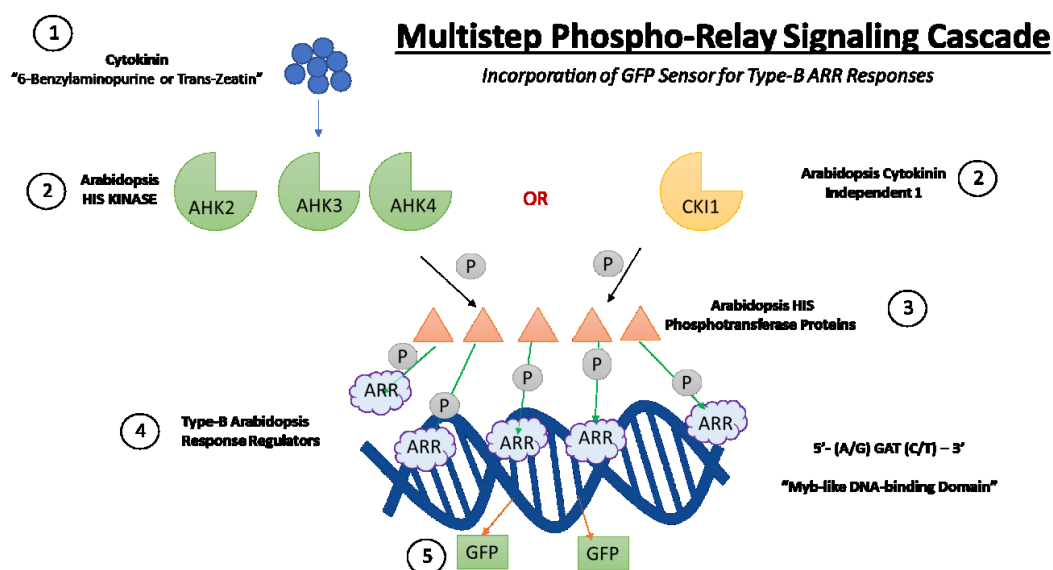


Figure 6.10 Creation of GFP Biological Sensor for Type-B ARR Responses. Through encoding a Myb-like DNA-binding domain promoter coupled with genetically encode GFP, the activity measurements of a multistep phospho-relay signaling cascade can be obtained, with specificity towards type-B ARR. (1-2) This cascade can be activated through cytokinin interactions with AHK2, (3) promoting the phosphorylation of a HIS Phosphotransferase Protein or through the same phosphorylation through CKI1. (4) The HIS phosphotransferase protein confers this phosphorylation upon the Type-B ARR which binds to promoters of target genes via their Myb-like DNA-binding domain and activates transcription and (5) downstream translation of GFP.

6.6.1 A Synthetic Sensor for Transcriptional Activity

The TCSn10 cell line was developed as an improved version of the reporter cell line TCS: GFP. Improvements were made to counteract the transgene silencing witnessed in progeny allowing an increase in generational stability of the GFP signal response [231]. This cell line has been genetically modified to allow comparative analysis of the multistep phosphor-relay signalling cascade in cells as characterised in *Figure 6.10*. Expression patterns of GFP can be seen in vivo as a reporter of the cumulative transfer of phosphoryl groups to type-B ARR. This allows transcription of GFP due to the coupling of a binding motive to a Myb-like DNA-binding domain. This cell line exemplifies biosensor exploitations, as the GFP is a quantifiable readout signal of cellular function.

6.6.2 MAC Chip Measurements

To determine the absolute GFP copy number produced by the transcription of type-B ARR, the GFP MAC chip methods, previously discussed in section 6.2.2 were implemented. To illustrate the diversity of the MAC chip technique protoplasts were isolated from the roots of the TCSn:10 cell line rather than the leaves. The average amount of molecular binding events captured from single cell experiments was $3.06 \times 10^4 \pm 2.2 \times 10^3$ as shown in *Figure 6.11 A*. This was the second highest GFP expressing cell line with an absolute GFP copy number of

$6.5 \times 10^5 \pm 4.6 \times 10^4$. However, as the GFP copy number for this cell line has no direct measurement upon protein copy number and only indicates the level of transcriptional activity the gamma distribution parameters do not present additional insight.

6.6.3 Epi-Fluorescence Measurements

TCSn10 root protoplasts were isolated and imaged upon a coverslip using the previously used parameters for image capture and background subtraction described in section 6.2.3. The average intensity for the root protoplasts was $5.81 \times 10^6 \pm 3.8 \times 10^5$. The epi-fluorescence images acquired for this cell line demonstrate a varied placement of the GFP reporter, such that it appeared in actin-like structures, the cytosol, or localised within vacuoles as previously represented in *Figure 6.3 B* and also displayed in *Figure 6.1 C*.

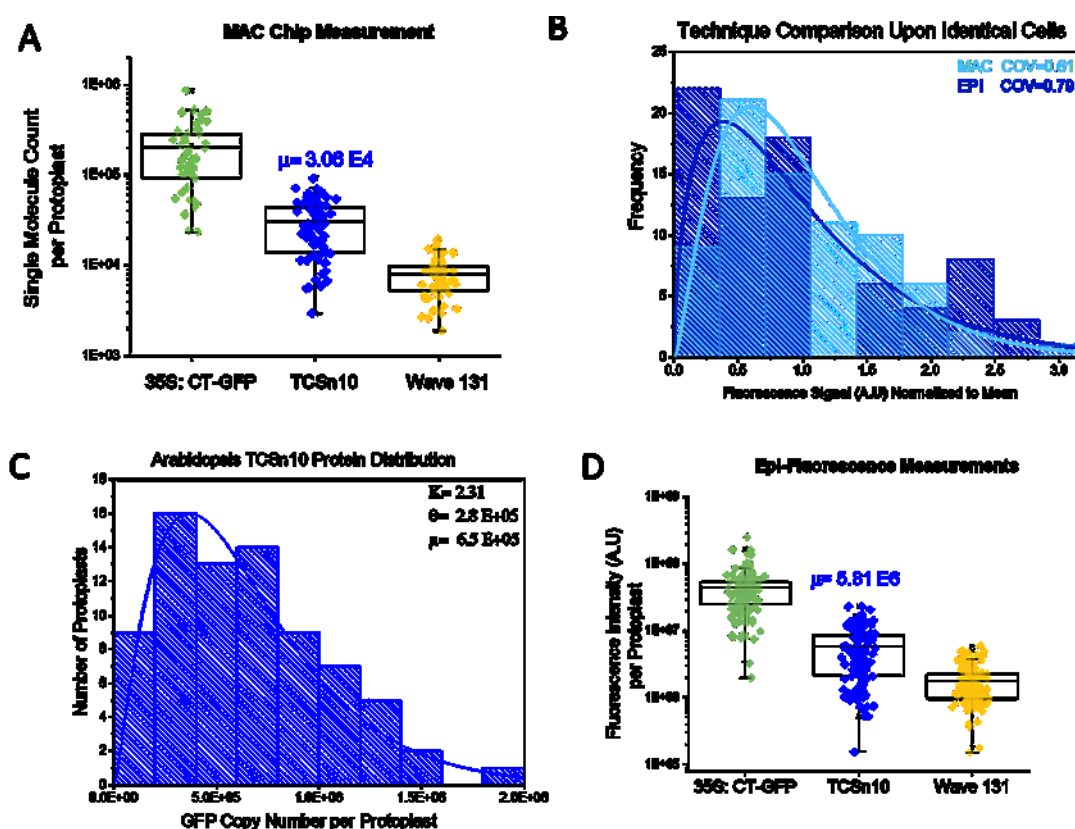


Figure 6.11 Compiled Measurements on TCSn10 Root Protoplasts. The TCSn10 cell line was the second highest fluorophore expressing cell line, with (A) MAC Chip measurements quantifying 3.06×10^4 GFP binding events. (B) Summating GFP intensity of the before lysis EPI-fluorescence images and comparison to single molecule binding events in the MAC chip for the same cell present a 77% similarity between the coefficient of variation (COV) for the two distributions. (C) Implementing the calibration curve of GFP binding events the absolute copy number of GFP per protoplasts is calculated to be 6.5×10^5 . (D) While the high-throughput epi-fluorescence measurements present an average fluorescence intensity of 5.81×10^6 .

6.7 Methodology Agreement

To be able to obtain absolute quantification of fluorophores within cells from epi-fluorescent microscopy the concordance between the two methodologies must be assessed.

6.7.1 Single Cell Precision Between Measurement Technologies

To assess the agreement between these two methodologies upon the same cell. Single cells were subjected to 2D epi-fluorescence measurement, followed by the MAC chip single molecule quantification. This direct comparison resulted in a Pearson correlation coefficient ($r = 0.88$) as shown in *Figure 6.12 A*, providing evidence of a relationship between the two measurements. The goodness of fit between these two measurements is ($R^2 = 0.77$).

However, correlation measurements do not provide insight into the systematic difference between the two methods. Therefore, to investigate the size of the disagreement between the two measurements, this data was converted to standardised z-score and plotted in a Bland-Altman diagram [238]. The difference between the two measurements produce a normal distribution. Where $\sim 5\%$ of the data ($n = 9$) is outside the limits of agreement while the remaining data points ($n = 174$) show close agreement *Figure 6.12 B*. This assessment presents evidence that there seems to be no systematic deviation between the measured values of the two techniques and the difference between the two measurements will be less than 0.1 in 95% of cases [239].

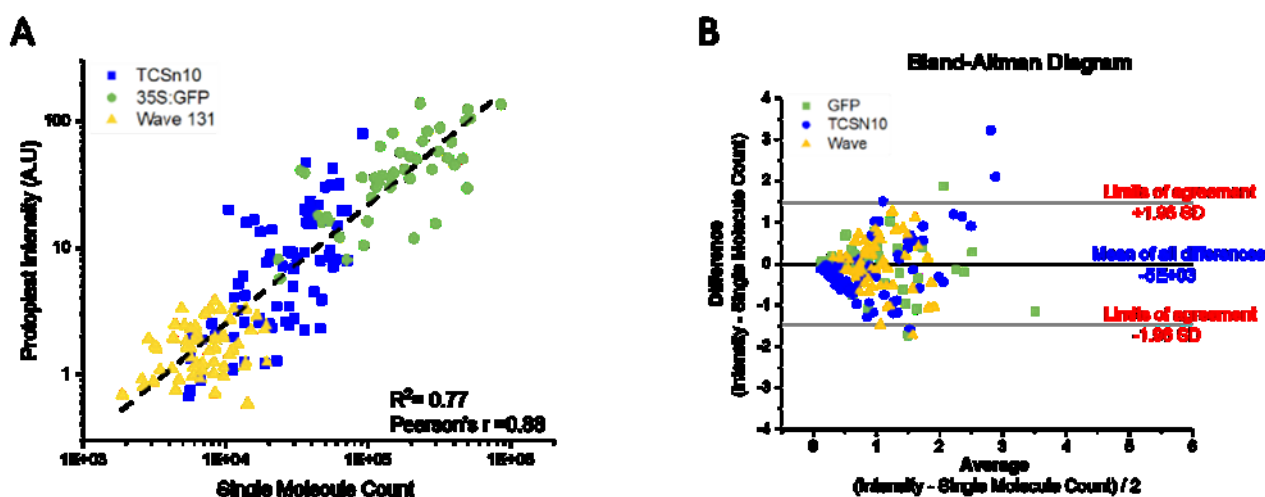


Figure 6.12 Same Cells Method Equivalence. Implementing the two measuring techniques on the same cell, while yielding different results at first glance, present a good linear relationship ($R^2=0.77$). (A) This relationship provides the possibility of predicting one measurement from the other one. (B) The relationship between these values were converted into a more informative presentation through a Bland-Altman diagram. Providing an assessment upon the systematic deviation from the mean-of-all-differences line.

6.7.2 Ratio Equivalence Between Measurement Technologies

The regulatory noise in protein expression is most commonly defined as the coefficient of variation. This noise leads to a technical challenge when comparing single-cell measurements by bulk measurements of coefficient of variation. Comparing this variation between these technologies, also called the F-test, as presented in *Equation 6.3*. Is a good initial test to compare precision, yet it cannot be used to assess the similarity in accuracy [240].

$$F = \frac{COV_{MAC}}{COV_{EPI}} \quad (6.3)$$

Therefore, we employ a method called the ratio of means to determine equivalency [241]. That is the dispersion of single-cell measurements and the technical noise generated by each measurement technology being outweighed by the importance of accurate mean expression measurements for the three total cell measurements compared between each technique.

This assessment is achieved by first comparing the intra-method means for all three cell lines, where each average measurement is compared producing three ratios of means factors representative of each technique presented in *Equation 6.4*. These three ratio of means factors were compared between the two techniques to determine the method equivalence, shown in *Equation 6.5*. Through implementing this method of comparing the intra-method ratio of means a percent method equivalence is calculated as 95.7% for $\frac{EPI_{RM}(GFP,WAVE)}{MAC_{RM}(GFP,WAVE)}$, 89.8% in $\frac{EPI_{RM}(TCSn10,GFP)}{MAC_{RM}(TCSn10,GFP)}$, and 86.0% for $\frac{EPI_{RM}(TCSn10,Wave)}{MAC_{RM}(TCSn10,Wave)}$. As these values were close to the maximum value of 1, as displayed in *Figure 6.13*, there is a high accuracy of measurement between these two techniques.

$$\begin{aligned} \frac{\mu(X)_{MAC}}{\mu(Y)_{MAC}} &= MAC_{RM}(X,Y) \\ &\text{and} \\ \frac{\mu(X)_{EPI}}{\mu(Y)_{EPI}} &= EPI_{RM}(X,Y) \end{aligned} \quad (6.4)$$

$$\frac{EPI_{RM}(X,Y)}{MAC_{RM}(X,Y)} = \% \text{ Method Equivalence} \quad (6.5)$$

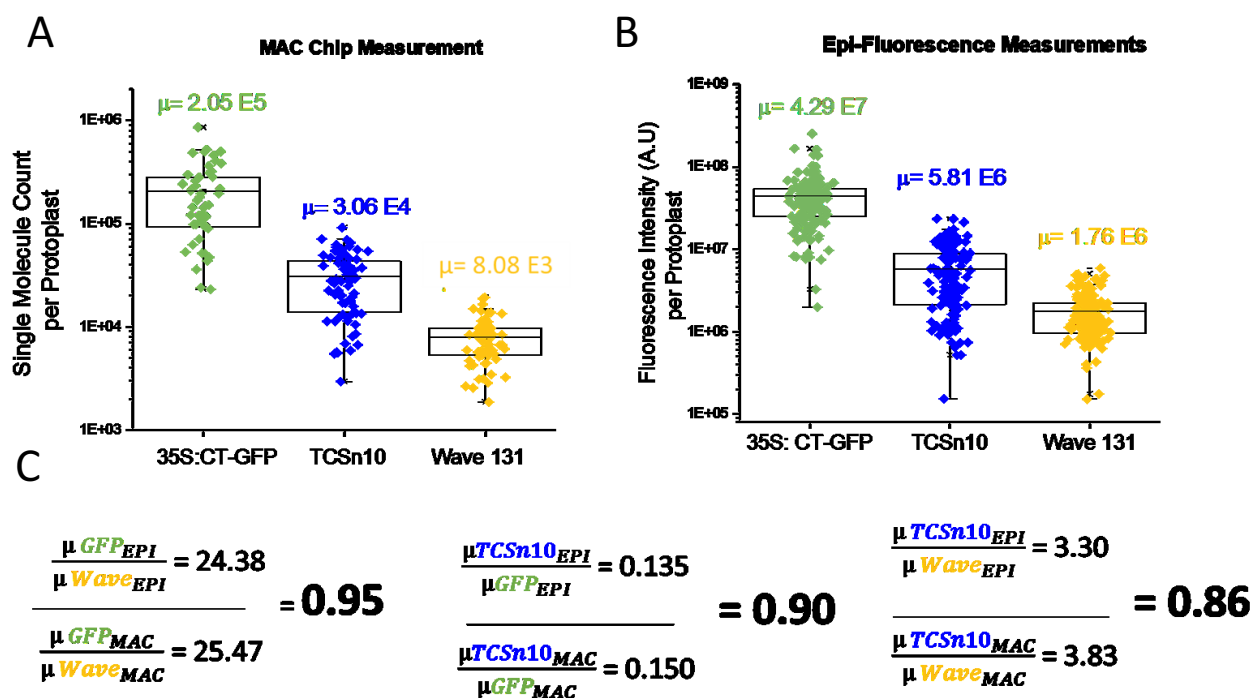


Figure 6.13 Differing Cells Method Equivalence Through Ratio of Means. Through the comparison of different cells measured by the (A) MAC chip technology and (B) epi-fluorescence microscopy a greater than 80% equivalence is presented. (C) This equivalence is quantified by comparison of the ratios of means factor between both technologies for two chosen cell lines.

6.8 Quantitative Epi-Fluorescence Validation

The ability to make epi-fluorescence microscopy quantitative is dependent upon not only the concordance between the two measurement techniques but also the application of a secondary standard within epi-fluorescence measurements. This secondary standard fluorescent intensity can be calibrated and then utilised to provide an absolute GFP copy number of neighbouring cells by determining the ratio of the GFP expressing cell to that of the secondary standard. Furthermore, this secondary standard can be employed upon other epi-fluorescent microscopes using similar light sources with identical excitation and emission filter sets.

6.8.1 Choice of Secondary Standard

A secondary standard for this purpose should ideally be a measurable quantity that shows little variance, is highly reproducible and effortlessly obtainable by colleagues. It is possible to use one of the three primary standards already discussed to calibrate other epi-fluorescence microscopes. However, as previously noted gene silencing in the offspring of genetically modified cell lines can be possible, providing erroneous measurements as a secondary standard. We have opted for a non-genetically modified approach to this situation

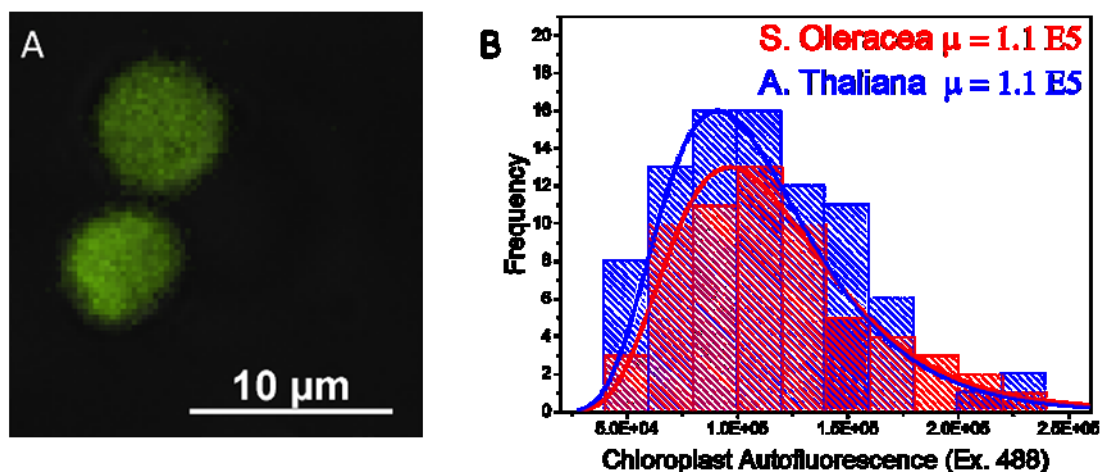


Figure 6.14 Chloroplasts Autofluorescence as a Secondary Standard for Quantitative Epi-Fluorescence Measurements. Chloroplasts are an ideal secondary standard, (A) first the small pixel area necessary would alleviate problems arising from space limitations as chloroplasts are $\sim 5 \mu\text{m}$. (B) Second the variation in the summated fluorescence intensity measurements is minimal, with respect to the three fluorescent cell lines measured, and appears to be reproducible across species.

by using the autofluorescence of spinach chloroplasts generated by excitation at 488 nm. This distribution can be seen in *Figure 6.14 B*. Chloroplasts, obtained from both *Spinacia oleracea* and *Arabidopsis thaliana* produce a highly reproducible fluorescence signal of $1.1 \times 10^5 \pm 5.2 \times 10^3$ with minimal variance, such that on average this variance is 1×10^5 fold smaller than the three previous fluorescent cell lines measured.

6.8.2 Linear Equation for Quantitative Epi-Fluorescence

Using the mean fluorescence intensity value from previously quantified cell lines and the mean fluorescence intensity of the secondary standard a ratiometric linear equation can be created, presented in *Figure 6.15 A*. This allows epi-fluorescent measurements to be translated, through the ratio of chloroplasts autofluorescence, into absolute copy numbers of GFP following a linear fit of ($Y = 1.21 \times 10^4 X + 9634$). An example of implementing this methodology can be seen in *Figure 6.15 B*, where a mixture of spinach chloroplasts and Wave 131 protoplasts have been pipetted upon a coverslip. The chloroplasts and protoplasts undergo fluorescence intensity quantification, where the specimen area of fluorescence intensity is summated followed by background subtraction of an identical area, previously presented as *Equation 6.2*, and produces a background subtracted fluorescence intensity for the object of interest (F_{obj}) [37].

The background subtracted measurements of protoplasts fluorescence intensity (F_x) is then divided by the chloroplast intensity (F_{chl}) producing a ratiometric comparison of fluorescence intensity ($\frac{F_x}{F_{\text{chl}}}$) which when incorporated into the linear equation ($Y = 1.21 \times 10^4$

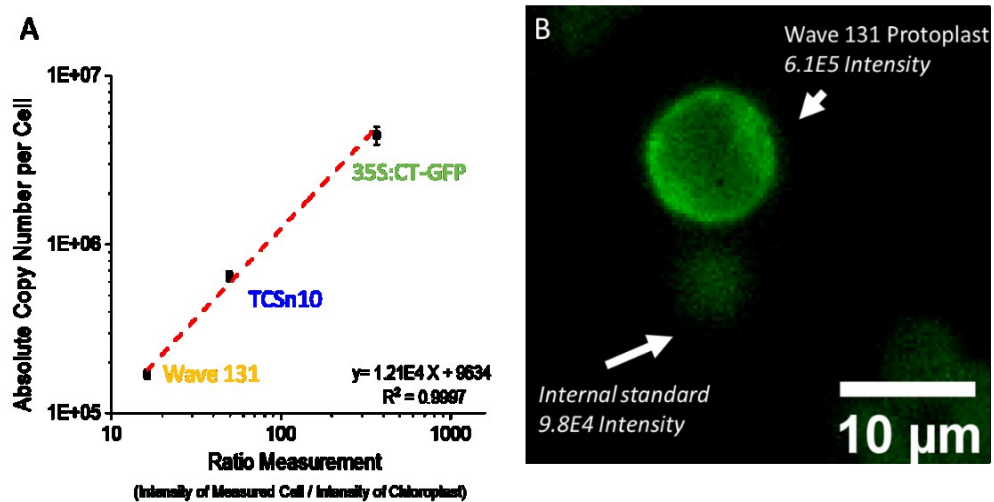


Figure 6.15 Implementing a Secondary Standard for Quantitative Epi-Fluorescence. (A) The ratiometric comparison of the mean fluorescence intensity values for the secondary standard (chloroplast) with that of three measured cell lines compared with the MAC chip technology measured absolute fluorescent proteins numbers presents a linear correlation ($R^2=0.99$) with a linear fit. For explanatory purpose of how to implement this linear fit. (B) The summated 2D fluorescent intensity of a specimen ($F_x = 6.1 \times 10^5$) is divided by the summated 2D fluorescent intensity of the internal standard ($F_{chl} = 9.8 \times 10^4$). This ratio measurement is ($\frac{F_x}{F_{chl}} = 6.224$), through implementing Equation 6.6 the specimen contains an absolute fluorescent copy number of ($P(x) = 8.5 \times 10^4$).

X+9634) provides the absolute copy number ($P(x)$) of the protoplast measured, descriptive of Equation 6.6.

$$P(x) = [1.21 \times 10^4] \left[\frac{F_x}{F_{chl}} \right] + [9.6 \times 10^3] \quad (6.6)$$

6.8.3 Proof of Concept Quantitative Epi-Fluorescence in Single Chloroplasts

To demonstrate the application of this method the absolute copy number of GFP was quantified per 35S: CT-GFP chloroplast. As the total copy number of GFP per protoplasts has already been quantified to be 4.5×10^6 and the average Arabidopsis protoplasts contain 29 chloroplasts we can estimate on average each chloroplast contains 1.5×10^5 GFP molecules. Also, it was previously identified by SDS-PAGE that in 35S: CT-GFP chloroplasts the GFP is approximately 1% of the total soluble chloroplast proteins [154].

Spinach chloroplasts and the 35S: CT-GFP chloroplasts were isolated by rupturing protoplasts by passage through a 10μm mesh filter. Ruptured and intact chloroplasts were separated using the Percoll gradient a previously discussed method in section 2.6.4. A 30 μL solution of the intact chloroplast solution was pipetted upon segregated areas of a coverslip, as shown in Figure 6.16 A. After chloroplast sedimentation, upon a coverslip coated with polylysine, epi-fluorescence images were acquired following the previously assigned parameters detailed in section 6.2.4. Images analysed for chloroplast fluorescence intensity and the ratios

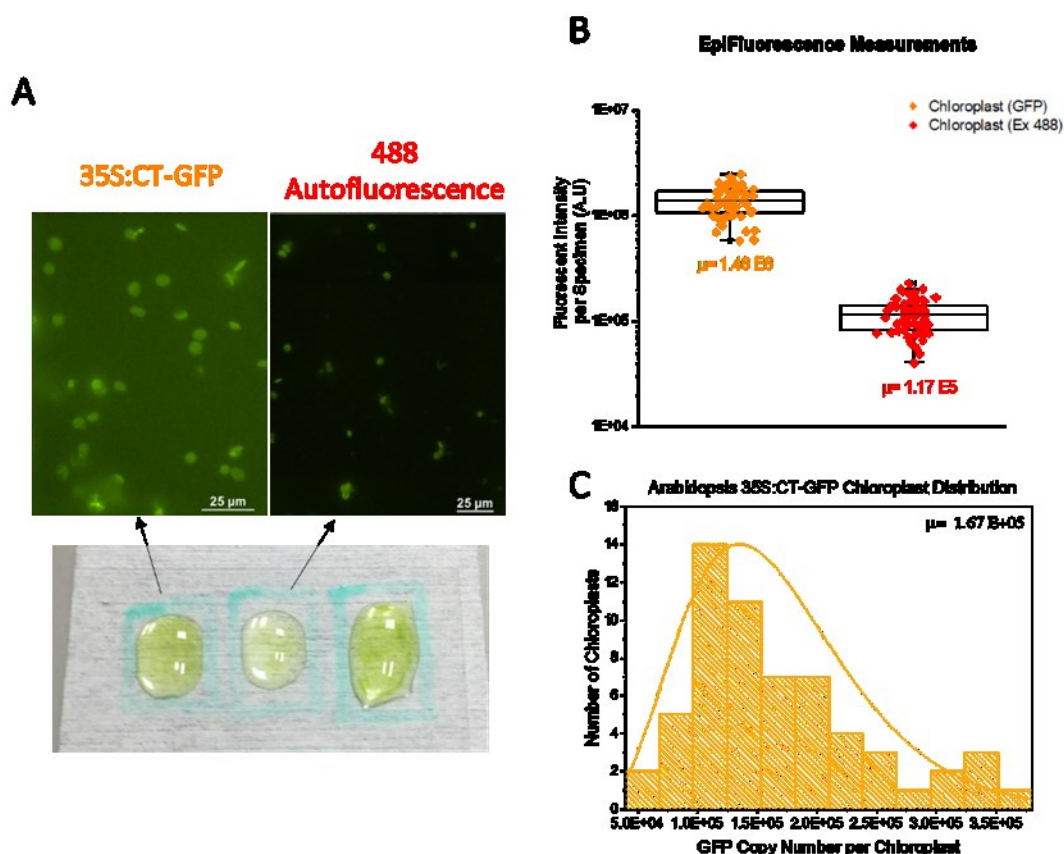


Figure 6.16 Proof of Concept Quantitative Epi-Fluorescence. (A) Pipetting a solution of GFP retaining chloroplasts in one area of a coverslip and placing wild-type chloroplasts in another area permits (B) comparative measurements on the integrated fluorescent intensity per chloroplast. (C) Applying Equation 6.6 to these measurement values a Gamma PDF distribution of GFP retention in the chloroplasts isolated from 35S: CT-GFP protoplast is produced, presenting an average fluorescent protein measurement of 1.67×10^5 .

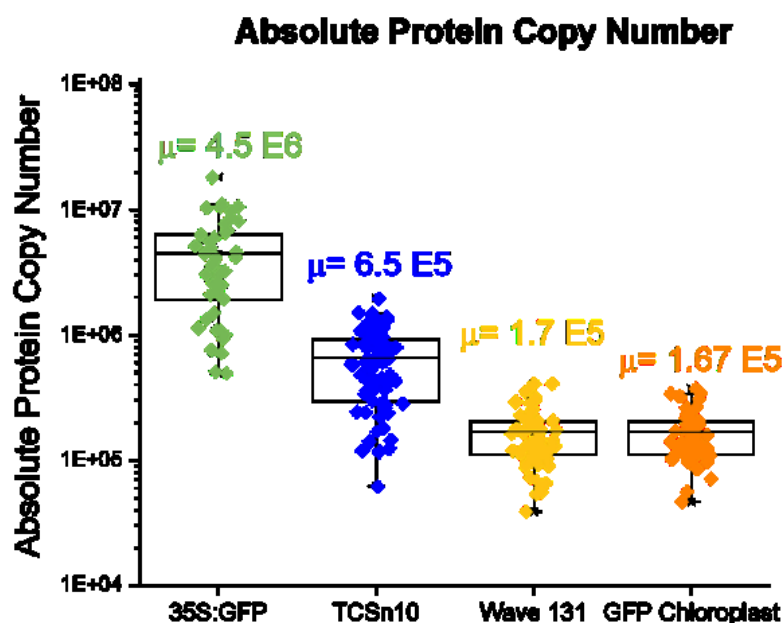


Figure 6.17 Absolute Fluorescent Protein Copy Number. The three fluorescent cell lines, 35S: CT-GFP, TCSn10, and Wave 131 measured through MAC Chip technology present an average copy number of 4.5×10^6 , 6.5×10^5 , and 1.7×10^5 respectively. The GFP retaining chloroplasts present an average copy number of 1.67×10^5 , this shows a 93% agreement with the aforementioned 35S: CT-GFP protoplast, if the average number of chloroplasts per protoplast is 29.

of the GFP expressing chloroplasts compared to the spinach chloroplasts autofluorescence was calculated as shown in *Figure 6.16 B*. This ratiometric comparison of fluorescence intensity was incorporated into *Equation 6.7* and presents the absolute copy number of GFP per chloroplast, this distribution is presented in *Figure 6.16 C*.

The average GFP copy number per chloroplasts is $1.67 \times 10^5 \pm 9.9 \times 10^3$. This measurement shows a 93% agreement with the estimated GFP copy number per chloroplast from the protoplast measurements. Which was 1.5×10^5 GFP molecules per chloroplast, if the average protoplast contains 29 chloroplasts, as previously measured in *Figure 2.16 D*. Further validity towards this measurement is also provided through this average GFP copy number constituting 1.19% of the total soluble chloroplast protein amount calculated from the information in section 2.3 and *Equation 3.1* and 3.2. This GFP per total chloroplast protein percentage agrees with the previously published measurements acquired by SDS-PAGE [154].

6.9 Conclusion

The addition of epi-fluorescence microscopy has provided a novel adaptation to further the throughput of the MAC chip absolute copy number measurement technology. It has been shown that through the ability of single molecule localisation we can obtain the distribution of single molecule intensities for GFP, allowing the quantification of binding events within the GFP bimolecular assay, presented in *Figure 6.6*. Through the creation of a calibration curve for the assay, the absolute FP copy number of three different *Arabidopsis Thaliana* cell lines were quantified. These three cell lines were further arbitrarily quantified for fluorescence intensity by epi-fluorescence microscopy.

Assessment of the concordance between the two measurement techniques for both the same cells and between different cells provided high levels of agreement. A high level of linear correlation (Pearson's $r = 0.88$) between the measurement techniques was witnessed for the same cell, as presented in *Figure 6.12*. The ratios of means between the two different techniques provide a greater than 80% similarity, shown in *Figure 6.13*. Since these measurement techniques show a high concordance, the implementation of a secondary standard, the autofluorescence from a spinach chloroplast excited by a mercury lamp from 465-495 nm, provides the ability to turn epi-fluorescence measurements quantitative through a ratiometric linear calibration line presented in *Figure 6.15*. We demonstrated the application of this technique by translating the fluorescence intensity ratio of 35S: CT-GFP

chloroplasts and the secondary standard into the absolute copy number of GFP within the chloroplasts. These GFP measurements show a 93% agreement with the estimated amount of GFP per chloroplast from the multiple chloroplasts within a protoplast measurement. The composition of GFP expression appears as 1.19% concerning total chloroplast protein content. This measurement is in good agreement with a previously published SDS-PAGE measurements.

In summary, the MAC chip may be inclusive for only researchers with resources to produce microfabricated devices. Upon the novel adaptation of this device towards permitting a quantitative fluorescence standard, the ability for protein quantitation can be widely available in all life science research laboratories implementing epi-fluorescence microscopy as a central laboratory tool.

Section 3:

Technology Evaluation:

Inferences on Cell-to-Cell Variability

Chapter 7: Sources of Noise in Gene Expression

Chapter 8: Conclusions and Future Work

Chapter 7

Sources of Noise in Gene Expression

This chapter examines the biological noise measured for the absolute protein copy numbers quantified in organelles, plants, and mammalian cells. We demonstrate that the average protein content does not correlate with the total noise in protein expression but does scale with the variance in protein expression. Associating the gamma distribution parameters with the central dogma of molecular biology, presents speculative insight towards four classes of dominant rates in protein expression, while the measured noise for Rubisco in protoplasts appears to be generated by chloroplast variability coupled with stochastic protein expression.

7.1 Introduction

The variation within a measurement is descriptive of noise and is quite often perceived as being undesirable [242]. Originating as a source of frustration in experimental set-ups and limiting our capacity to quantify low signals results in experimental scientists spending a great deal of time attempting to control and dampen this noise. However, biological systems are intrinsically heterogeneous (i.e. biologically noisy) arising from the stochastic fluctuations of the molecular processes that control cellular regulation. Producing a noisy environment for experimental scientists, regulated through evolution, which has been fine-tuned and optimised to manifest significant phenotypic differences to promote survival, as occurs with certain viruses, bacteria, and even cancer cells [243]. In contrast, evolution also provides stability against fluctuation through noise-dampening mechanisms [244], essential for controlling the production of cell differentiation, such as the cellular constituents which compose a plant root. The development of experimental platforms to quantitatively measure this biological noise will help provide an answer to the contrasting question, provided by the above examples, as to what amount of noise constitutes beneficial or harmful effects to a cell and what level of noise can be tolerated?

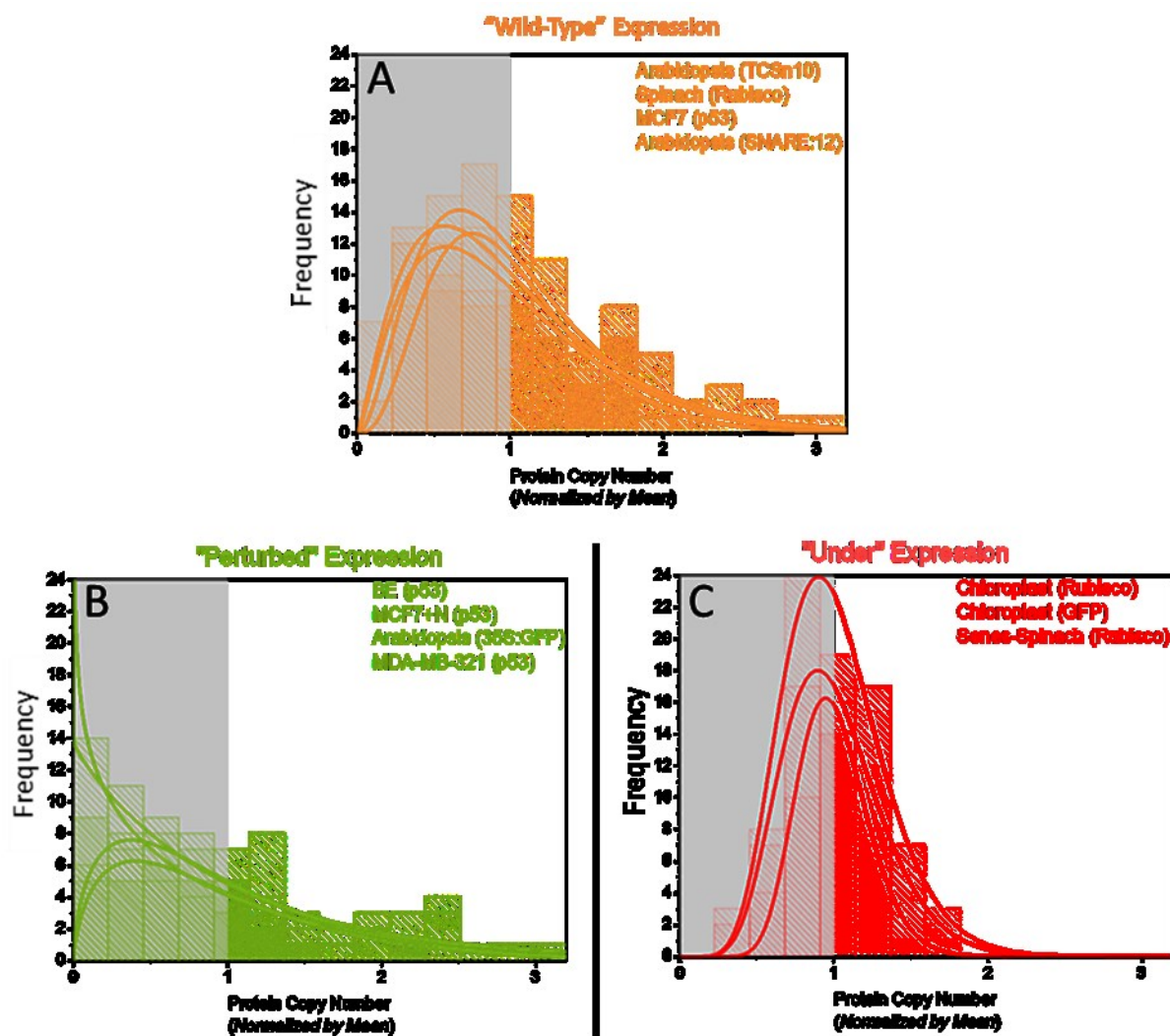


Figure 7.1 Visual Comparison of the Variation in Expression Distributions. Through a preliminary visual inspection of the 11 proteins discussed within this thesis and fit to a Gamma distribution PDF. A variation with respect to normalized copy numbers appears to show a reproducible trend with respect to the distributional shape. A trend in distributional shape that can be designated as (A) protein expression under wild-type conditions, (B) under perturbed conditions and (C) the under-expression proteins which represent measurements in either single organelles or under heightened protein degradation.

A central theme to the technologies presented within this thesis has been the ability to assess cell-to-cell variability (i.e. variance) for protein copy numbers with an absolute resolution. This variance, concerning gene expression, arises from stochastic bursts of transcription and translation, encoding a wealth of information about the rates of production and degradation central to a proteins existence [70]. As presented in section 1.5, the steady-state protein copy number distributions (i.e. a product of the translation rate) have been acquired through population measurements, that is multiple cells measured to infer the temporal protein fluctuations within an individual cell, a theory rooted in the ergodic hypothesis. The measurements of the mean and the variance of the protein copy number quantifies the total noise in protein expression, a quantity that is a summation of both

intrinsic and extrinsic noise sources concerning the cellular process of protein expression. Furthermore, it has been presented that the parameters of the gamma distribution can describe this cell-to-cell variability. Using this previously mentioned knowledge and absolute noise measurements of protein expression from both *E. coli* (Taniguchi et al.) and *S. cerevisiae* (Newman et al.) [86, 89]. This chapter attempts to examine the noise in protein expression quantified for over ten orders of magnitude.

7.2 Measuring Noise in Protein Expression

Initial assessment of the steady-state protein copy numbers presents evidence that (1) distributions are skewed to the right with a varied kurtosis dependent on the protein measured. (2) The shape of the distributions (variance) appears to identify underlying perturbations to the process of gene expression. Visually displayed as the perturbed proteins, seen in *Figure 7.1 A*, showing an expression deviating from the reproducible standard deviation to mean relationship of the expressed wild-type proteins, seen in *Figure 7.1 B*. (3) Single organelle measurements, presented in *Figure 7.1 C*, exhibit a 2-fold decrease in variation comparative to the same proteins measured in single protoplasts. (4) Also, we find no apparent correlation between the measured squared coefficient of variation and the average copy number ($R^2 = 0.12$) seen in *Figure 7.3*.

7.2.1 Modelling Transient Gene Expression

The intrinsic noise of protein expression can best be described by the stochastic events identified within the central dogma of molecular biology. These events may be defined by a simple two-state model consisting of mRNA creation (k_1), a simple Poissonian event produces one mRNA molecule, coupled with mRNA degradation (γ_1) producing an amount of mRNA which undergoes translation to create protein (k_2). This distribution of manufactured protein is further regulated by protein degradation rates (γ_2). However, this two-state model only provides Poissonian or Gaussian distributions which poorly approximates the variance in comparison to experimental results [245]. Through integration of the initiation event, descriptive of promoter fluctuations (k_{on}/k_{off}), and allowing bursty transcriptional processes (more than one mRNA is created in each initiation event), this three-state model, as shown in *Figure 7.2*, presents a long-tail distribution similar to experimentally measured distributions for eukaryotic data [71, 77].

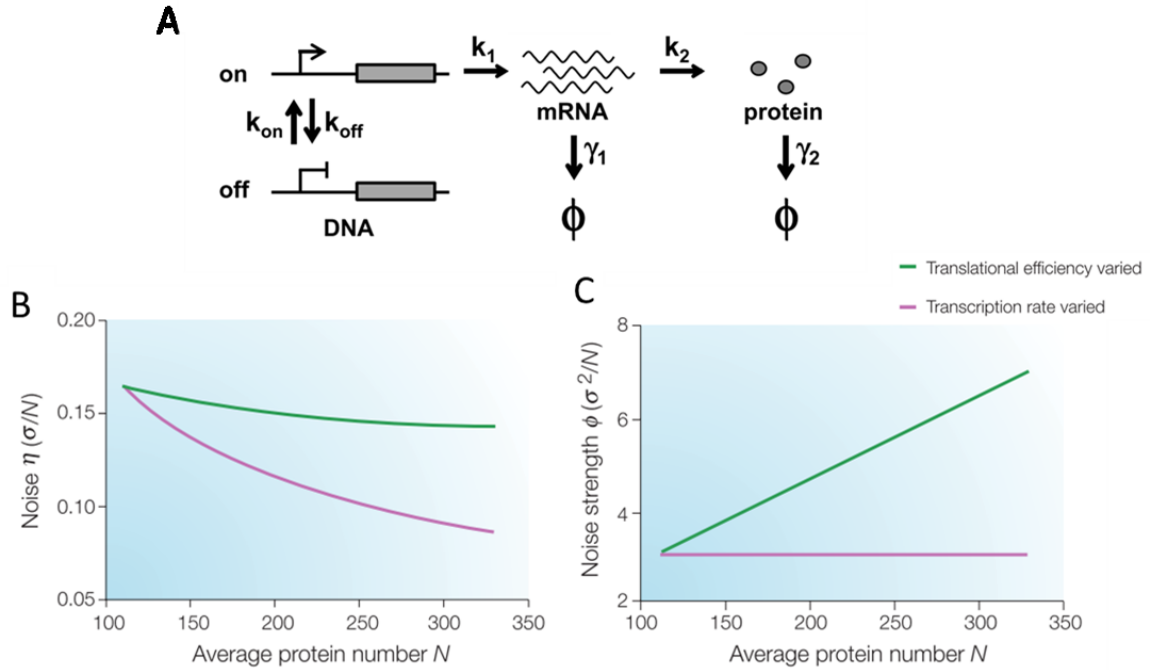


Figure 7.2 Modeling Approaches for Protein Distributions. (A) The promoter transitions between an inactive “off” state and an active “on” state with rates k_{on} and k_{off} . During the on state, mRNAs are transcribed at the rate k_1 , and proteins are translated at the rate k_2 . mRNA’s and proteins are degraded at the rates γ_1 and γ_2 , respectively. The state of degraded mRNAs and proteins are indicated as ϕ . The predicted dependencies of either the noise (B) or noise strength (C) upon production of the average protein copy number, through increasing parameters in the three-state model changing either the translational or transcriptional efficiencies. Reproduced from [245].

The importance of adding this parameter to the model was later shown at a single molecule level that indeed eukaryotic organism undergo convoys of RNA polymerases, if under a viral promoter, which produce multi-scale mRNA bursting upon a single k_{on} event [246]. While the exact opposite approach appears in bacteria, where a greater amount of k_{on} events occur with less mRNA transcription per event. Dar et al. also showed that in eukaryotes mRNA creation is an equally modulated event (1:1) between the frequency of k_{on} events with the multi-scale mRNA bursting [247].

The extrinsic noise is descriptive of variables that affect the entire network which generates the intrinsic noise measurement. The protein expression three-state model reproduced for each cell is equally impacted through cell cycle effects, upstream network contributions, and alterations in environmental factors [248]. Vahid et al. has modelled contributions and provided evidence that extrinsic noise impacts the distribution of protein expression in a non-trivial manner, showing that upon the addition of extrinsic noise a measured distribution becomes wider (larger σ) than what is expected by a Poisson distribution [248, 249].

7.2.2 Gamma Distribution Relationship with Gene Expression Mechanisms

These distributions of cell-to-cell variability have been proven through both theory and experiments to be characteristic of a gamma distribution [73]. This statistical distribution arises naturally concerning the waiting times between Poissonian distributed events, as previously discussed in section 1.5.3 and is defined as

$$x ; \text{Gamma} (K, \theta); p(x) = \frac{x^{K-1} e^{-x/\theta}}{\Gamma(K)\theta^K} \quad (7.5)$$

Where the probability of observing a protein copy number in a single cell, $p(x)$, is descriptive of the shape parameter K and the scale parameter θ . These parameters can be related to the waiting times between the biochemical events that compose the three-state model that facilitate the creation of cell-to-cell variability in protein copy numbers [85]. That is defined as K is the ratio of protein degradation (γ_2) to mRNA creation (k_1) and is descriptive of the *burst timing*, while θ is defined as the ratio of protein creation (k_2) to mRNA degradation (γ_1) and is descriptive of the *burst size*.

$$K = \frac{\gamma_2}{k_1} \quad \& \quad \theta = \frac{k_2}{\gamma_1} \quad (7.6)$$

the mean and the variance of the gamma distribution can be defined by

$$K\theta = \mu \quad \& \quad K\theta^2 = \sigma^2 \quad (7.7)$$

Therefore, these gamma distribution parameters which contain biochemical rates can be approximated to the descriptive characteristics of distributions, where the noise η^2 is defined as

$$\eta^2 = \frac{\sigma^2}{\mu^2} \approx \frac{K\theta^2}{(K\theta)^2} = \frac{1}{K} = \frac{k_1}{\gamma_2} \quad (7.8)$$

while the noise strength FF_p is defined as

$$FF_p = \frac{\sigma^2}{\mu} \approx \frac{K\theta^2}{K\theta} = \theta = \frac{k_2}{\gamma_1} \quad (7.9)$$

7.2.3 Quantifying Noise Sources in Protein Expression

Through multicoloured experiments, where two fluorescent proteins are under identical regulatory sequences, researchers can distinguish between intrinsic and extrinsic noise. This technique was originally applied by Peter Swain, for characterising the intrinsic and extrinsic noise in *E. coli* protein expression [62]. Studies using this method in alternative organisms, such as *A. thaliana* have identified the same trend [87]. That extrinsic noise is a dominant contribution for abundant proteins. While intrinsic noise, the random timing within the three-state model, is only dominant when a lower copy number of proteins are manufactured. The intrinsic and extrinsic noise measured for constitutive abundant protein expression was $\eta^2 \approx (0.058 + 0.09)$ and $\eta^2 \approx (0.04 + 0.09)$ for *E. coli* and *A. thaliana* respectively. This presents a lower limit of noise in constitutive abundant protein expression representative of ($\eta^2 \approx 0.148$) for *E. coli* and ($\eta^2 \approx 0.13$) in *A. thaliana*.

Performing absolute proteomic quantification of fluorescent fusion proteins in *E. coli* and *S. cerevisiae* it is reaffirmed, with absolute values, that extrinsic noise is a dominant source when protein copy numbers are < 10 proteins per cell. This lower noise limit was measured as $\eta^2 \approx 0.09$ in *E. coli* and $\eta^2 \approx 0.095$ in *S. cerevisiae*. When the protein copy number is at a low expression (> 10 protein per cell) the intrinsic noise is dominant and can be characterized as simple Poisson production and degradation of mRNA and protein [85].

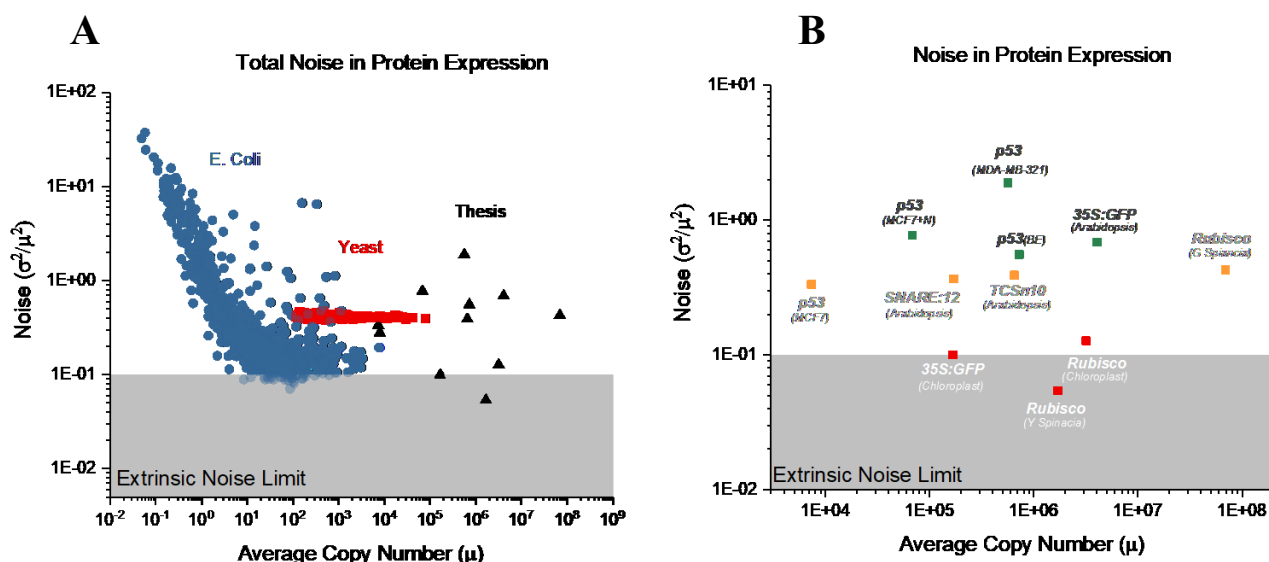


Figure 7.3 Measurements of Total Noise in Protein Expression. Previous experiments have provided limitations found in expression noise designated as an intrinsic ($1/\mu$) and extrinsic (0.1) noise limit. Proteins expressed under normal expression rates exhibit consistency in expression noise above this extrinsic noise limit, while protein distributions from organelle measurements appear in close proximity. Upon perturbation of protein expression through protein mutations, viral amplification or senescence we see an increase or decrease in total expression noise.

This is characterized as the intrinsic limit upon protein distributions measurements and can be approximated by

$$\eta^2 \approx \frac{1}{\mu} \quad (7.10)$$

This measurement of intrinsic noise was reaffirmed in *S. cerevisiae* by Bar-Even et al., through performing measurements upon yeast cells that shared identical size and granularity. Permitting the removal of the extrinsic noise arising from cell cycle effects from the total noise measurement. Allowing Bar-Even and colleagues to see a linear correlation of $\eta^2 \approx \frac{1}{\mu}$ within the intermediate protein abundance, while higher copy numbers presented deviation from the behavior of poissonian statistics [88].

7.2.4 Summary of the Noise Measurements in Protein Expression

Summarising all these experiments upon protein noise provides insight that for the total noise measurements (η^2) of the gamma-distributed cell-to-cell variance. (1) The ability to measure intrinsic noise from total noise is only possible under low copy number protein expression (>10 copies per cell) where intrinsic noise shows Poissonian scaling ($\eta^2 \approx 1/\mu$). (2) The total noise in protein expression will never be lower than ~ 0.09 for abundant proteins (<100 copies per cell). (3) The reason for this consistency of noise in abundant proteins is a dominant contribution of extrinsic noise.

7.3 Variance to Mean Scaling Behavior in Protein Expression

If the total noise in protein expression (η^2) can be treated as constant, one would expect the variance to scale with the mean. As presented in *Figure 7.4*, there is indeed a global scaling for protein expression between the *E. Coli*, Yeast, and this thesis' data ($\sigma^2 = \mu^2$). This equality has been modelled by Birtwistle et al. using the gamma distribution parameters, showing that this correlation between the standard deviation (σ) and the mean (μ) can present a constant noise if the shape parameter [$K = (\gamma_2/k_1)$] is constant and the scale parameter [$\theta = (k_2/\gamma_1)$] is changed to drive alterations in mean protein levels [43]. Explained with respect to the gamma distribution as

$$\mu = K\theta; \sigma^2 = K\theta^2; \rightarrow \sigma = \left(\frac{1}{\sqrt{K}}\right)\mu \quad \therefore \quad \eta = \frac{\sigma^2}{\mu^2} = \frac{1}{K} \quad (7.11)$$

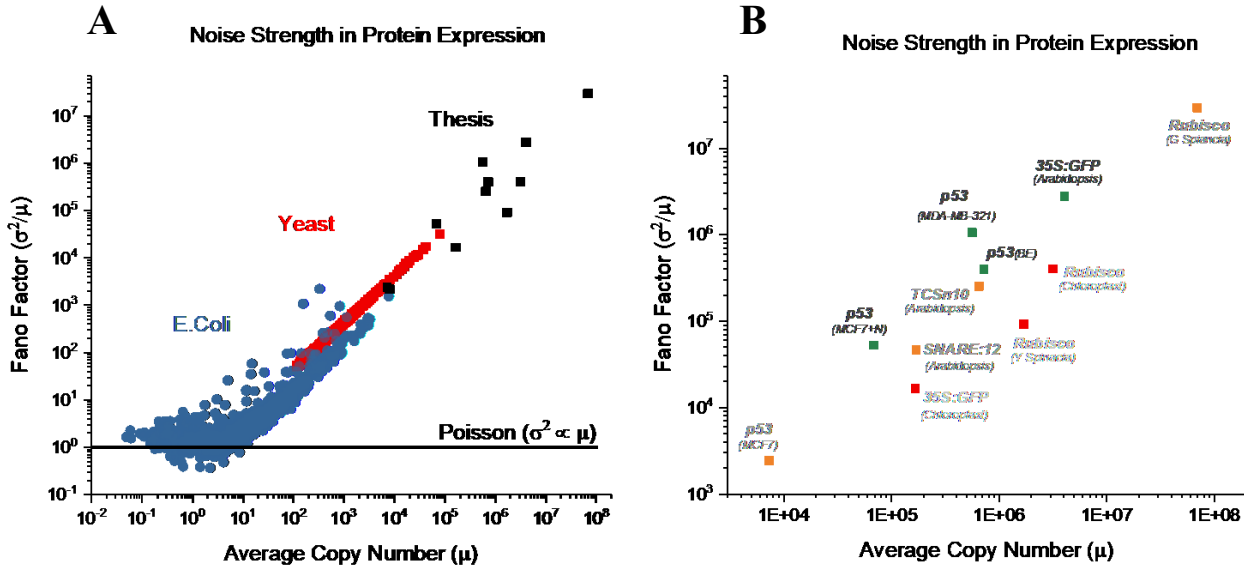


Figure 7.5 Noise Strength in Protein Expression. The noise strength (σ^2/μ) is a measurement of the deviation from a Poisson distribution. The low copy number E. coli proteins show linearity similar to that of the Poisson noise, while higher copy number proteins deviate from this limit. Such that the increased bursting events in transcription and translation resulting in a greater noise strength which correlates with the average protein copy number.

The steady-state measurements for protein distributions were dependent upon the number of mRNA molecules present and show a distribution much larger than a Poisson distribution identified as

$$p = \frac{k_1 \left[\frac{k_2}{\gamma_1} \right]}{\gamma_2} \quad \text{and} \quad \frac{\sigma_p^2}{\mu_p} = \frac{\left[\frac{k_2}{\gamma_1} \right]}{1 + \left[\frac{\gamma_2}{\gamma_1} \right]} \quad (7.13)$$

Through Thattai et al. modelling single gene expression (without extrinsic noise) it can be seen that by varying the number of bursts of proteins from a mRNA transcript ($\frac{k_2}{\gamma_1}$) a correlation of Fano Factor with average protein content can be seen. Thattai et al. inferred that using this model intrinsic noise is controlled at the translational level as the non-poissonian variance exhibited in protein distributions, measured by the Fano Factor, could be attributed to the translational process. Yet this can only be true if the steady-state variance in mRNA production follows a Poissonian distribution. However, through single molecule FISH experiments upon mRNA, it has been proven mRNA production in abundant proteins (>10) is not a Poissonian distribution.

7.4 Noise Creation in Protein Expression

The ability to define and quantify the exact processes which manipulate the noise measured within the protein distributions would require a greater number of multiplexed measurements upon each cell, compared to the measurements obtained within this thesis. Exemplified by Albayrak et al. solving for the stochastic two-state model of the central dogma after absolutely quantifying joint mRNA/protein distributions [252]. Also presented by the ability to identify protein degradation rates as a dominant mechanism between the measured p53 abundances in BE and MCF7 cells, made possible by average measurements of mRNA levels [221]. As no measurements of mRNA abundance were performed on the cells in this thesis the ability to extrapolate rates from the attributed shape and scale parameter, would only be possible under large assumptions. Therefore, through the literature and Equations 7.5-7.9, we can approximate the inverse of (K) to be a ratio of mRNA creation and protein degradation and the scale parameter (θ) to be a ratio of protein creation of mRNA degradation. Through plotting the inverse of the shape and the scale parameter, using the data obtained in this thesis, it appears this graph can be descriptive of how the central dogma rates attribute to the noise in protein expression as shown in *Figure 7.6*.

7.4.1 Non-Perturbed Expression (II.A)

The diagram presented in *Figure 7.6* visualizes the high reproducibility of the K parameter in the gamma distribution measurements of un-perturbed protein expression within the blue region of the diagram, labeled II.A. It appears with respect to the x-axis that the rate of protein degradation for these proteins is always greater than double or double the rate of mRNA creation ($1/K = 0.5$). Concerning the y-axis, as previously recognised the process of protein creation is far greater than Poissonian expression ($\frac{\sigma^2}{\mu} = 1$), as all of the proteins measured present numbers far greater than 1 on the y-axis. This is representative of the bursting behavior within the transcription and translational processes.

7.4.2 Perturbed Expression (II.B-III)

As the noise in protein expression increases, the measured distributions shift into new regions, characterised by the shape and scale parameters. It appears this shift into the II.B region presented in *Figure 7.6*, is descriptive of a decrease in protein degradation rates or an increase in mRNA creation rates where ($1/K = < 0.5$). As previously mentioned by measuring average mRNA content in both MCF7 and BE cells it has was presented that protein degradation is a dominant mechanism for the measured noise in p53 expression. When

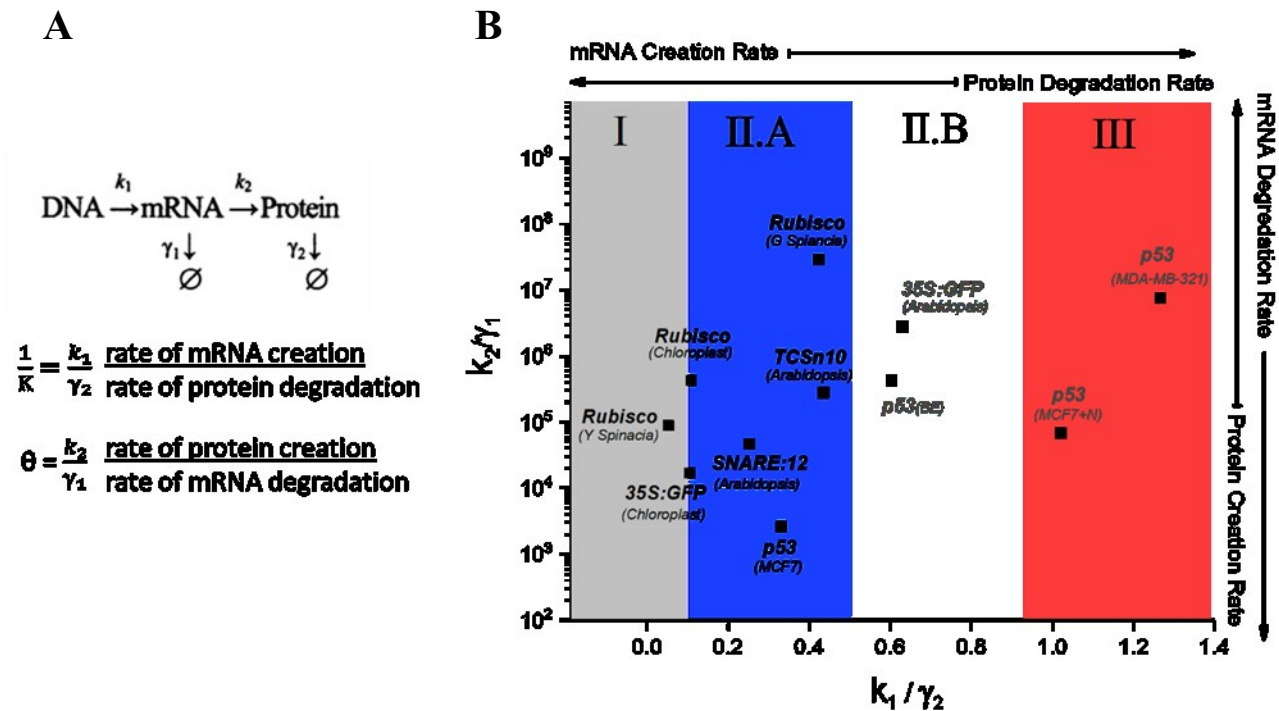


Figure 7.6 The Ratio of Rate Orders for Noise Creation. To describe the noise in protein distribution measurements, (A) the parameters of the gamma distribution can be assessed as a ratio of the rates associated with the central dogma of molecular biology. (B) Plotting the inverse of the shape parameter (x-axis) with the scale parameter (y-axis) four classes, representative of the measured total noise can be designated. Noise measurements residing under the extrinsic noise limit were designated as (I). A majority of non-perturbed high copy number (<100) proteins reside within this region (II.A). As protein degradation rates decrease or mRNA creation rates increases the value for $(1/K)$ becomes greater than 0.5 producing the regions of (II.B) and (III).

protein degradation equally matches the rate of mRNA creation ($1/K = 1$) or the rate of protein degradation is less than that of mRNA creation ($1/K < 1$) noise in protein expression moves into the (III) region. Validation of this explanation can be appreciated through the knowledge that the addition of Nutlin inhibits the feedback between MDM2 and p53 causing the degradation rates of p53 to be dramatically reduced [210].

7.4.3 Extrinsic Noise Limit (I.)

As the diagram, presented in *Figure 7.6*, has provided agreement with previous knowledge towards protein degradation, it presents a novel perspective that as protein degradation rates increase the measured noise in protein expression would fall below the noise produced by cellular divisional events (the extrinsic noise limit). Through the single cell and organelle measurements upon Rubisco, we present experimental evidence towards describing the extrinsic noise limit as both descriptive of the cell cycle noise and the processes that maintain steady-state protein levels (k_2/γ_2).

The first example of this is representative of cell senescence and provides experimental evidence that translational bursting (k_2) coupled with cell cycle noise allows

protein measurements to be above the region demarcated as (I), as measured for Rubisco content in healthy mesophyll cells. As discussed in section 3.4.2, upon cell senescence, the protein Rubisco undergoes increased degradation and the general gene expression program is shut down [114]. Presenting the distributions for Rubisco in senescent mesophyll cells to show an increase in protein degradation, showing the modification of the Rubisco distribution from the region of (II.A) into (I).

The second example is from the single organelle measurements and shows evidence that cell cycle noise is part of the extrinsic noise limit. As previously discussed in section 3.5.4, measurements of the noise in Rubisco expression presented two populations of chloroplasts. These two populations can be characterised such that the second population has undergone a greater number of divisional events comparative to the first population. Furthermore, as Rubisco is a highly expressed protein composed of large (chloroplast encoded) and small (nuclear encoded) subunits, conferring high regulation upon protein content, exemplified as a high reproducibility in expression variance. Similar to the variance found in abundant proteasomal genes in yeast and bacteria.

As presented in *Figure 7.7*, the noise in Rubisco expression for the 1st chloroplast population may be composed of low-level extrinsic noise (ex. variability in transcription factor abundance, variability in stroma volume) coupled with the intrinsic noise of network

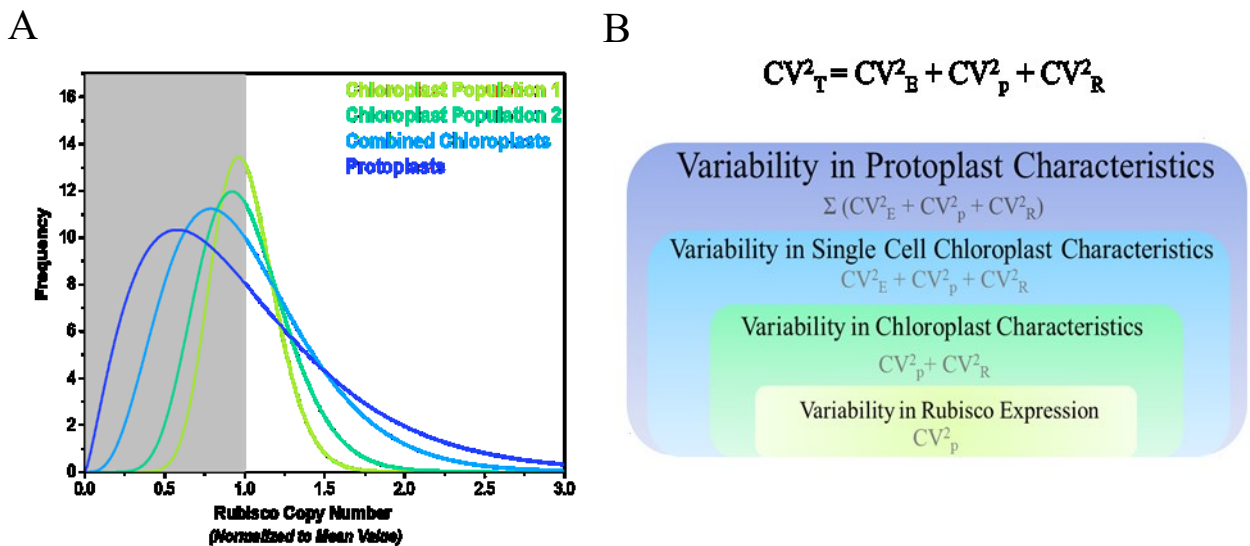


Figure 7.7 Noise Generation Through Chloroplast Divisional Events. (A) The measured total noise (CV_T^2) in Rubisco content increases with respect to the complexity of the organism measured. Chloroplast population 1 exhibits little variability and can be considered a limit for protein expression noise (CV_P^2). Chloroplast population 2 has undergone a division of Rubisco content thereby adding partitioning noise (CV_R^2). A single protoplast through development contains both sets of chloroplasts, due to asynchronous chloroplast division, this is characteristic of a cell cycle noise (CV_E^2). Therefore, a single protoplast contains all three noise sources for Rubisco expression ($CV_E^2 + CV_P^2 + CV_R^2$) and summation of these single protoplasts provide the final measurement of Rubisco Noise in a population of protoplast (B).

fluctuations in Rubisco production (CV^2_P) creating a noise of $CV^2_P = 0.04$. After division, the 2nd population incurs an additive extrinsic noise penalty, partitioning noise (CV^2_R), which raises the noise to $CV^2_P + CV^2_R = 0.08$. As chloroplast division occurs asynchronously a single protoplast would contain both populations, thereby causing the noise to include the random chloroplast-division events (CV^2_E) producing $CV^2_E + CV^2_P + CV^2_R = 0.18$. Upon the summation of the chloroplast variability, shown in *Figure 7.7 B* the Rubisco content within each protoplast $\Sigma (CV^2_E + CV^2_P + CV^2_R) = 0.43$, presents as a noisier distribution that resides within the non-perturbated protein region of (IIA).

Providing possible evidence that the extrinsic noise limit is a formation of the intrinsic noise of protein creation coupled with general cell cycle noise. In protoplasts, the observed cell-to-cell variability in chloroplast measured protein copy numbers is a summated measurement much greater than just single cell stochastic expression [253]. Therefore, measurements of expression noise for photosynthetic proteins could be an unresolved measurement of chloroplast variability in combination with the stochastic protein expression.

$$CV^2_T = CV^2_E + CV^2_P + CV^2_R \quad (7.14)$$

7.5 Conclusion

Through implementing protein quantification technologies, the ability to obtain protein distribution measurements for a variety of organism have provided some insight towards this measured signal to noise ratio. It may be concluded that the signal is representative of the average content per translational bursts, while the noise can be described by the relationship between protein and mRNA stability coupled with cell cycle division events. However, the inability to extrapolate the rates within the three-state model of protein creation, which can be descriptive of the shape and scale parameter, limits the ability to determine the dominant rate concerning noise creation within the measured protein distributions.

It does appear that the average copy number measured shows a high correlation with the Fano Factor or noise strength, as shown in *Figure 7.5*. A correlation that could be interpreted as the ratio of protein creation (k_2) with respect to mRNA degradation (γ_1) determines the absolute copy numbers we have measured. While the noise around this

absolute copy numbers appears descriptive of the inverse of the shape parameter ($1/K$), presenting evidence that the total noise (σ^2/μ^2) measured in absolute protein copy numbers is dependent upon alterations in either protein degradation (γ_2) or mRNA creation (k_1).

Provided by these measurements, it is apparent that the stochasticity of protein expression within plants is a rather reproducible process in comparison to that found in the creation of drug-resistant bacteria or cancer cells, as recently highlighted by Araújo et al. [87]. Even though protein expression is reproducible the measurements of chloroplastic proteins highlight the creation of measurement errors in single cells by the amplification of noise arising from divisional organelle events. To truly summate the extrinsic noise contribution from organelles, single organelle measurements are key and would allow greater measurement accuracy in protein expression. These measurement implications have been highlighted previously in mitochondria through both advancements in technology and a greater understanding of organelle-level heterogeneity [253, 254].

Chapter 8

Conclusions & Future Work

The work in this thesis was initially targeted towards advancing the gap in quantitative plant biology, with specificity towards the ability to achieve single-cell resolved proteomic measurements with absolute quantification. Section 1 detailed the attainment of this initial goal in both bi and tri-molecular assays and the recognition of its limitations in antibody dependence and analysis throughput. Novel technologies carried out to overcome these restrictions, provided in section 2, are either in the form of microwell development or the creation of a secondary standard permitting absolute fluorophore quantification in epifluorescence microscopy. In section 3 an overall assessment of the thesis' measured cell-to-cell variability in protein expression presents insight towards scaling between the variance and mean, resulting in evidence that the total expression noise of a protein does not scale with the mean copy number.

8.1 Section One Conclusions

The quantification of cellular variability, originally a philosophical afterthought, has now transitioned to an expansive area of research. This transition is in part due to an ongoing revolution in miniaturisation technologies promoting the assessment of single cell 'omic heterogeneity, illuminating what was previously invisible in bulk measurement technologies. While these single-cell approaches have been employed in a growing number of animal studies, a gap remains in their application for quantitative plant biology. This research gap presented an opportunity, explored through section 1, in promoting a different perspective for plant biology by employing a miniaturised technology for assessing single plant cells.

8.1.1 Chapter 2: Basic Principles & Techniques

Upon the combination of fundamental microfluidic, fluorescence, and proteomic principles a micro total analysis system titled the Microfluidic Antibody Capture chip was created addressing the aforementioned research gap in single plant cell biology. This platform is composed of 50 analysis chambers, and CFD modelling provided evidence that chambers

were optimized for limiting protein diffusion and inhibiting chamber to chamber contamination. Each assay chamber contains a microprinted capture antibody spot, which permits either bi or tri-molecular assays for protein (antigen) quantification. Further exploration within the methods of capture antibody spot fabrication presented a direct relationship between the concentration of capture antibody printed and the amount of antigen captured, while the choice of printing buffers or printing additives influenced antibody capture performance and spot-to-spot reproducibility. We further provide evidence towards an alternative technique of enhancing antigen capture through a reduction of the microfluidic assay chamber volumes.

The incorporation of single plant cells into a microfluidic device often employs hydrodynamic trapping structures or electrical manipulation. For the first time, we present the ability to implement optical tweezers for the precise manipulation of protoplasts in a microfluidic environment, followed by optical lysis upon the chamber sequestered cells. Fascinatingly, as no other literature has explored this workflow, we find that optical lysis only dissociates the protoplasts cell membrane allowing the liberation of chloroplasts and the nucleolus. While originally this was perceived as disadvantageous, due to the addition of a secondary lysis step, it later became appreciated as a method for additional quantification of the number of chloroplasts per protoplast.

Upon the combination of these techniques preliminary experiments were carried out; first to characterise the contribution of autofluorescence from the lysis of single mesophyll plant cells, and second to assess the precision of this novel quantitative plant biology platform. The implementation of both TIRF microscopy and a single molecule counting algorithm allowed the definition of autofluorescence contribution to be quantitatively minimal under the excitation of a 488 nm laser line. The precision of this platform was assessed through comparative measurements upon GFP expressing *Arabidopsis thaliana* mesophyll cells. Widefield fluorescence images were taken before the event of cellular lysis; this protoplast fluorescence intensity was directly compared to the post-lysis fluorescence intensity of the bimolecular GFP pull-down assay implemented within the microfluidic device. We find that a reduction in chamber volume from 4.5 nL to 0.75 nL increased the total antigen capture by 52% while increasing the correlation between the two fluorescence measurements methods from ($R^2 = 0.80$) to ($R^2 = 0.88$). In summary, this chapter provided evidence of a novel plant protein quantification platform that can faithfully reproduce quantitative measurements with similar precision to widefield epi-fluorescence measurements, in turn advancing the gap in quantitative plant biology.

8.1.2 Chapter 3: Single Cell to Organelle Rubisco Quantification

While we offered evidence of a working micro total analysis system in Chapter 2 for the field of plant biology, it is hard to fathom the benefits or appreciation for this device which only arbitrarily measures fluorescent proteins. As only a handful of native proteins expressed within the protoplast present fluorescent properties and arbitrary units of measurement are meagre and unsatisfactory during this era of absolute digital quantification. In response to those criticisms, this chapter involves designing an absolute quantification assay for native non-fluorescent protein identification.

The non-fluorescent protein Rubisco was chosen as the target antigen, as a source of purified Rubisco was available and previously published bulk analysis methods present that Rubisco constitutes up to 50% of the total soluble protein within chloroplasts. As previously identified in Chapter 2 a reduction in chamber volume would return a higher percentage of antigen bound. Therefore, only the sub-nanoliter chambers were implemented, and a novel tri-molecular assay for the absolute quantification of Rubisco was optimised. This tri-molecular assay, after calibration, presented a linear range of quantification upon the addition of 1×10^5 to 1×10^8 Rubisco molecules per nL and was applied for the quantification of Rubisco content in both healthy and senescent single mesophyll protoplasts isolated from Spinach oleracea. The average healthy cell Rubisco content was $6.91 \pm 4 \times 10^7$ followed by a 20-fold reduction in Rubisco content $1.70 \pm 0.4 \times 10^6$ during senescence. This reduction has previously been confirmed, as mesophyll cells degrade Rubisco to redistribute nitrogen to younger and developing leaf tissue. However, with this single cell approach, we can not only identify mean expression levels but also ascertain a novel perspective in noise levels (variance-to-mean relationship) within these populations. We find, when Rubisco expression is under non-perturbed conditions, the expression noise is 0.28. This noise is above the proposed extrinsic noise limit of 0.1 and while Rubisco expression is under the perturbed conditions of degradation the expression noise falls below the extrinsic noise limit to 0.053.

This single cell approach also provided a new perspective on the relationship between leaf development and Rubisco expression. As the age of a protoplast increases (i.e. the protoplast diameter increases) a maintained concentration of 7.69 ± 2 mM is reached. Furthermore, we present a higher correlation between the Rubisco copy number with the number of chloroplasts present within a protoplast ($R^2=0.91$), rather than that of the protoplast diameter ($R^2=0.84$). Implying evidence that protoplasts “tune” into this ideal Rubisco concentration for photosynthesis at a level of chloroplast regulation. This evidence highlighted an apparent need, that to truly understand the mechanisms that were driving

Rubisco expression and this noise associated, the organelle responsible for the final step in Rubisco assembly would need further analysis.

The single chloroplast measurements presented an average Rubisco copy number of $3.18 \pm 1 \times 10^6$ and after performing GMM fitting upon these multiplexed measurements of both the Rubisco copy number and organelle volume, two organelle subpopulations were identified, for the first time. Population 1 is composed of chloroplasts containing an average Rubisco concentration of 0.576 M, while Population 2 contains an average concentration of 0.236 M. Further assessment of a possible relationship between chloroplast age (i.e. the number of divisional events), identified by cpDNA content, and Rubisco concentration presented a high correlation ($R^2 = 0.95$), suggesting organelle subpopulation formation may arise from chloroplast divisional events. A novel approach was devised in measuring multiple chloroplasts extracted from a single cell to explore this theory further and provided a comprehensive experiment towards mesophyll cell development and its association with Rubisco content. From this experiment, we conclude throughout protoplast development the chloroplastic content shifts from originally containing a Population 1 majority of chloroplasts into containing a majority of Population 2 chloroplasts. In summary, this analysis of chloroplast variability within the protoplast, hidden from previous bulk analysis methods, demonstrated that the aforementioned “tuning” could be associated with asynchronous chloroplast division events permitting the maintenance of a catalytically optimal Rubisco concentration within the protoplast.

Further insight into Rubisco content through protoplast development was gained through the creation of a unique multiplexed antibody assay to assess, in arbitrary units, the protein-protein interactions of Rubisco and Rubisco Activase. This assay measured a higher correlation between Rubisco and Rubisco Activase in photosynthetically active mesophyll cells ($R^2 = 0.70$), in comparison to the relationship witnessed in senescent mesophyll cells ($R^2 = 0.15$). A lack of correlation in the senescent cells appears to arise from a greater number of Rubisco Activase molecules binding to Rubisco, an association hallmarked by either Rubisco denaturation or a decrease in the presence of ATP.

8.1.3 Chapter 4: Bulk Cell Analysis of 4-HPPD

With success in developing a microfluidic platform for single plant cell proteomic measurements, as presented in Chapter 2. Followed by the systems beneficial transition towards absolute quantification of a non-fluorescent native protein, detailed in Chapter 3. It became apparent that to promote further advancements in quantitative plant biology a protein with critical quantitative motivation would need to be chosen. 4-HPPD was this

protein, due to its existence as a crucial herbicide target, and its lack of quantification either by bulk or single cell analysis methods.

By selectively screening multiple antibody combinations in an open chip microarray format, a tri-molecular assay for 4-HPPD quantification was achieved. The chosen assay consisted of an identical monoclonal antibody for both capture and detection of 4-HPPD. Providing superficial evidence that 4-HPPD is composed as a complex. Furthermore, multiple approaches to increasing the antigen capture of this assay were undertaken. We present that the best approach was creating favourably orientated capture antibodies, where Biotin is conjugated to the F_c region of an antibody, facilitating a higher amount of actively binding antibodies within the printed spot and in turn, a 2-fold increase in antigen capture compared to the unmodified assay.

Even after these multiple alterations, the integration of this assay for *Arabidopsis thaliana* mesophyll cell analysis presented pitfalls, representative of limitations in single cell assay detection. To surpass this assay limit multiple mesophyll protoplasts were loaded by optical trapping into chambers, yet no signal was identified. Therefore, we turned our attention to bulk cell lysate, consisting of 1×10^6 cells/mL (~ 100 protoplasts per analysis chamber). This protoplast suspension was vacuum infiltrated into assay chambers and the antibody spots measured presented an average signal corresponding to 271 ± 98 4-HPPD molecules per mesophyll cell. Furthermore, as mRNA levels for 4-HPPD rise during etiolation, a second experiment was performed to assess the 4-HPPD content in visually identified etiolating leaves. This experiment, following identical methodology, presented an average signal corresponding to 975 ± 100 4-HPPD molecules per mesophyll cell.

8.2 Section Two Conclusions

The establishment of the first known miniaturised technology targeted for single plant cell absolute protein quantification, described in section 1, presented multiple limitations. For example, in Chapter 3 the implementation of optical tweezers for protoplast isolation caused tedious and time-consuming experiments with a minuscule return on experimental throughput. On average 25 protoplasts were analysed per experiment. Comparing this with an ability to rapidly isolate and measure 45 chloroplasts per experiment, it is evident that a bottleneck existed concerning the manipulation of larger cell volumes. Another constraint, presented in Chapter 4, is the limited accessibility of antibody assays which can provide the requisite limit of detection for the quantification of single cell protein copy numbers. A third

limitation arises from the exclusivity of this technique, only researchers with the capability of producing microfabricated devices would benefit from this approach. These limitations motivated the continued work, described in section 2, towards implementing novel approaches to overcome these restrictions.

8.2.1 Chapter 5: A Reduced Volume Microwell Array

Microwell arrays have the potential for being powerful platforms in performing biological measurements due to the confinement of molecules in small volumes, permitting a higher local concentration of the analyte to be measured. Through the combinatorial approach of implementing closed volume microwell arrays with a p53 antibody assay, we established a novel microfluidic device, titled the Flip Chip. We initially presented the advantages of implementing the Flip Chip through a comparison of volume dependent p53 assay calibrations curves. This comparison presented that a reduction in chamber volume and an increase in antibody capture spot diameter produces a higher concentration of antibody, and in return provides a higher antigen capture percentage. Specifically, this was demonstrated by the 1.75 nL Flip Chip displaying an antigen (p53) capture percentage comparable to that of a 0.75 nL MAC Chip. This novel closed volume approach presented an opportunity to address the constraint, identified in Chapter 4, for improving the limit of detection (i.e. the limit of detection decreases) if the affinity of an antibody assay is not suitable for quantification at the level of single cell analysis.

To further investigate assay detection limit improvements, the p53 assay was modified through altering the detection antibody through the choice of the conjugated fluorophore. We find that conjugating the detection antibody with AlexaFluor 488 compared to AlexaFluor 647 presented a 3.2-fold difference for antigen capture. That is 22.6% and 5.37% respectively when executed in a bimolecular assay in a volume of 5 nL. These values were determined from the dissociation constant obtained by SPR measurements. However, when comparing these fluorophore alterations in a tri-molecular assay, in a volume of 5 nL, containing an identical capture antibody. We find a 1.1-fold difference in antigen capture, that is 3% and 1.47% for AlexaFluor 488 and AlexaFluor 647, respectively. Calculated from the dissociation constant obtained in 4.5 nL MAC chip calibration experiments. Presenting evidence, for the first time experimentally, and in agreement with our previously determined theoretical approaches that the choice of capture antibody and its affinity towards the antigen is the dominating effect in tri-molecular assay detection.

To optimise the platforms operational procedure for single cell proteomic evaluation. Preliminary assessment of a 25 chambered microarray was performed to assess the p53

content in a colorectal cancer cell line. We concluded that the optimal technique consisted of a microwell wetting transition from a Cassie state into a Wenzel state, followed by wiping with a coverslip at a 45° angle to introduce an advancing contact line for cell seeding. From this experiment, we presented a proof of concept result, a correlation ($R^2 = 0.88$) between the number of colorectal cancer cells present in a chamber and the measured antibody spot fluorescence. This validation facilitated the motivation for fabricating a larger microarray that consisted of 300 chambers to increase experimental throughput.

Experiments to evaluate the throughput of this large-scale, 1.75 nL chambered microarray involved quantifying the p53 content of two breast cancer cell lines. The first cell line, MCF-7, is a wild-type p53 expressing cell line and presented an average p53 content of $7.3 \times 10^3 \pm 547$ with an expression noise of 0.35. The second cell line, MDA-MB-231, an overexpressing mutant p53 cell line was quantified, for the first time, to contain on average $5.6 \times 10^5 \pm 1.03 \times 10^5$ p53 molecules with an expression noise of 1.88. Fascinatingly, this large expression noise was accompanied with 76 times more p53 in comparison to the previously measured wild-type p53 expressing cell line. The microarray technique was compared with 4.5 nL MAC chip experiments, which after the creation of a 4.5 nL p53 calibration curve, permitted the quantification of the previously assessed colon carcinoma cell line and an MCF-7 cell line treated with Nutlin. Containing on average $7.23 \pm 0.87 \times 10^5$ and $6.81 \pm 0.78 \times 10^4$ p53 molecules, with an expression noise of 0.55 and 0.77, respectively.

Upon comparison of these platforms, the microarray was advantageous in operational workflow, as the loading of single cells into chambers was accomplished in under ten minutes. Compared to the thirty minutes necessary for cell isolation through optical trapping in the 4.5 nL MAC chip experiments. However, a disadvantage to the Flip Chip operational protocol is the limitation in single cell capture. As increasing the concentration of cells to increase cell capture events leads to a disadvantageous increase in the before assay background binding events. This limitation allows the user of the device to trap cells quicker, yet the throughput of single cell analysis is similar to that of one MAC chip experiment.

8.2.2 Chapter 6: Quantitative Fluorescence Microscopy

Absolute quantification in 'omic measurements, is an area of research containing multidisciplinary benefits, such as assessing the rates of biological processes between research laboratories, quantifying the general architecture of cellular structure, providing comparative evidence for assessing pharmacological responses and producing precision in parameters for mathematical modelling. These benefits were the basic motivation for a future in which absolute quantification of biomolecules could easily be shared between cross-

disciplinary research and interdisciplinary colleagues. However, this future is hindered by access to technologies for the quantitative assessment of cellular functions, and those who have access, underutilise their data sets by only reporting qualitative data or even arbitrary units. The work in this chapter, initially motivated in addressing the limitations of the microfluidic platform in throughput and proficient antibody assays, resulted in a novel calibrated secondary standard for quantitative epi-fluorescence which presents an opportunity to excel towards this future of sharing absolute values.

The creation of the secondary standard was rooted in the performance of a bimolecular antibody assay for fluorescent protein quantification. The assay was calibrated with a known amount of GFP and presented the ability for absolute quantification over three orders of magnitude. The assay was implemented in the MAC chip and permitted the fluorophore quantification of three transgenic *Arabidopsis thaliana* cell lines. Specifically, single mesophyll cells from the 35S: CT-GFP cell line and Wave 131 cell line, quantified as $4.5 \pm 0.5 \times 10^6$ and $7.9 \pm 0.5 \times 10^3$ fluorophores per cell, respectively. While single root cells from a TCSn:10 cell line, selected to present this platform's ability for cell-type versatility, were evaluated to contain $6.5 \pm 0.4 \times 10^5$ fluorophores per cell. Following from the insight obtained in Chapter 2, that is the microfluidic platform can faithfully reproduce quantitative measurements with similar precision to widefield epi-fluorescence measurements. The three transgenic cell lines were quantified for fluorophore content in the 2D image acquired through epi-fluorescence microscopy.

A greater than 80% agreement between the two measurement technologies is demonstrated. This was determined by comparing the intra-method means for all three cell lines, which generated three ratio of means factors for each measurement technology. The three ratio of means factors were compared between the two techniques and led to a final assessment of method equivalence. The concordance between the two techniques permitted, for the first time, the creation of a novel epi-fluorescence secondary standard, the autofluorescence generated by a chloroplast. This organelle was chosen as it presented minimal fluorescence signal variance, being 1×10^5 fold smaller than the three previous fluorescent cell lines. A ratio of the fluorescence intensity was taken for this secondary standard and the three previously measured transgenic cell lines. As the absolute fluorophore content was known for those three cell lines, a ratiometric linear calibration line was produced which would permit the absolute fluorophore quantification of an unknown cell by measuring a simple fluorescence ratio between the unknown and the secondary standard.

The validity of this procedure was assessed through a proof of concept experiment. Epi-fluorescence images were acquired for chloroplasts isolated from 35S: CT-GFP protoplasts. A fluorescence ratio was taken for these GFP containing chloroplasts with respect to the secondary standard, and the ratiometric calibration line determined the average GFP copy number per chloroplasts to be $1.67 \times 10^5 \pm 9.9 \times 10^3$. This GFP measurement presented a 93% agreement with previous bulk chloroplast measurements (i.e. the single 35S: CT-GFP protoplasts measurand by the microfluidic platform). As the average Arabidopsis protoplast contains 29 chloroplasts presenting the estimate that each chloroplast would contain 1.5×10^5 GFP molecules.

While the MAC chip technology may only be inclusive for researchers with resources to produce microfabricated devices. This novel adaptation from the device, permitting a quantitative fluorescent secondary standard, showcases the ability for protein quantitation to be widely available in all life science research laboratories that implementing epi-fluorescence microscopy as a central laboratory tool.

8.3 Section Three Conclusions

Multiple technologies were developed in this thesis for facilitating single-cell protein measurements. The initial target was the development of a platform for single plant cell protein quantification. Recognition of the limitations within this technology led to synergistic approaches, resulting in a microarray platform and a quantitative fluorescence secondary standard. Implementing these technologies resulted in numerous absolute protein copy number measurements expressed within organelles, plants, and mammalian cells, presented in *Figure 8.1*. Notably, these single-cell measurements provided insight towards the cell-to-cell variability in protein expression (i.e. biological noise). It was the recognition of this biological noise, which led to the motivation for Chapter 7. Assessing not only this thesis data but also measurements from previously published quantitative proteomic data in both *E. coli* and *S. cerevisiae* to assess biological noise on a global scale.

8.3.1 Chapter 7: Sources of Noise in Gene Expression

The stochastic process of gene expression results in the presence of cell-to-cell variability in single-cell protein measurements. This biological noise comprised of intrinsic and extrinsic noise sources that arise respectively within the production process or generated by environmental factors associated with the process. Assessing the current literature provided

insight into how the total noise in protein expression is a product of limitations produced by intrinsic and extrinsic noise sources. That is (1) an ability to measure intrinsic noise from total noise is only possible under low copy number protein expression (>10 copies per cell) defined by intrinsic noise showing Poissonian scaling ($\eta^2 \approx 1/\mu$). (2) The total noise in protein expression would never be lower than ~ 0.09 for abundant proteins (<100 copies per cell). (3) The reason for this consistency of noise in abundant proteins is a dominant contribution of extrinsic noise.

Initial assessment of this cell-to-cell variability for the proteins measured in this thesis presented evidence that (1) distributions are skewed to the right with a varied kurtosis dependent on the protein measured. (2) The shape of the distributions appears to identify underlying perturbations to the process of gene expression. (3) Single organelle measurements exhibit a 2-fold decrease in variation comparative to the same proteins

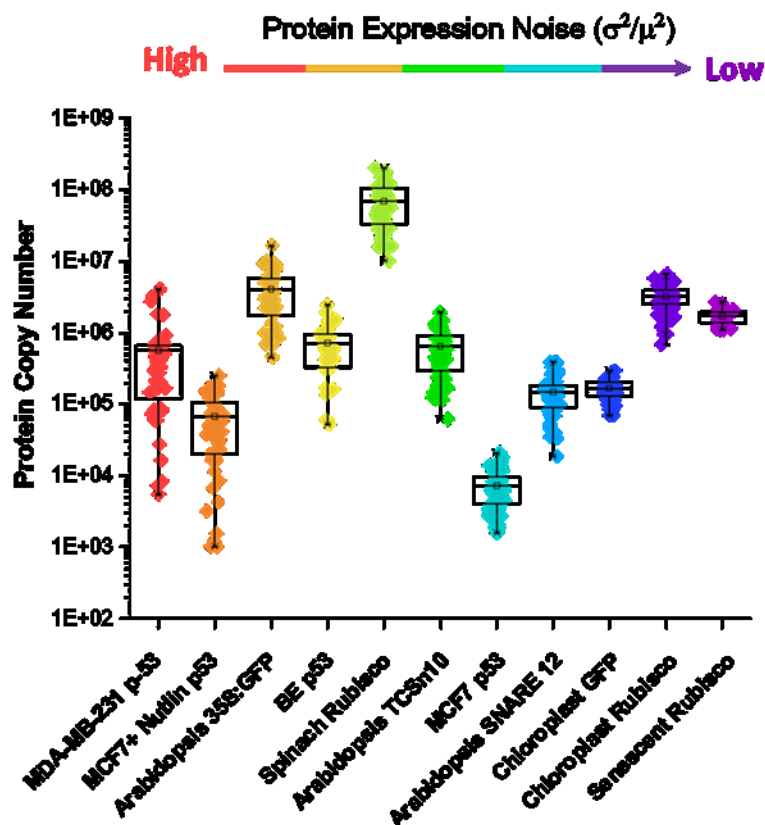


Figure 8.1 Protein Expression Variation. The capabilities of the technology presented in this thesis have allowed protein expression measurements by implementing three different antibody assays. We have measured the expression of 35S: GFP, TCSn:10, and SNARE12 in *Arabidopsis thaliana* through an anti-GFP bi-molecular assay. Previous and collaborative efforts have allowed a tri-molecular assay with anti-p53 antibodies to measure the expression of p53 in four cell lines MDA-MB-231, MCF7, BE, and MCF7 with the addition of Nutlin, while the implementation of anti-Rubisco antibodies in a tri-molecular assay have allowed Rubisco measurements in *Spinacia oleracea* from single cells to organelles. Copy number measurements acquired in these different cell types, for over six orders of magnitude, present a large variation in expression noise measured to range from (1.89 to 0.05).

measured in single protoplasts. (4) Interestingly, we find no apparent correlation between the expression noise and the average copy number ($R^2 = 0.12$) for proteins measured over six orders of magnitude. However, we do find that the average copy number scales with the variance ($\sigma^2 = \mu^2$). Comparison of our data with measurements from previously published proteomic data sets in *E. coli* and *S. cerevisiae* presented an identical scaling factor to the one identified above. Indicating a global trend between the variance and mean in protein expression. Assessing the noise strength, a measurement of the deviation from a Poisson distribution which would scale as ($\sigma^2 = \mu$), shows that low copy number *E. coli* proteins behave as a Poisson distribution. While higher copy number proteins deviate from this Poisson distribution presenting a higher noise strength which can be explained as an increase in bursting events in transcription and/or translation [248].

To determine the dominant contributor to this noise strength, the rates associated with the central dogma of molecular biology were approximated through their relationship with the gamma distribution of the measured proteins cell-to-cell variability. Specifically, this was defined as *burst timing*, where the parameter K is the ratio of protein degradation (γ_2) to mRNA creation (k_1) and the *burst size*, where the parameter θ is defined as the ratio of protein creation (k_2) to mRNA degradation (γ_1). We find that only performing measurement on the product of the translation rate creates an inability to uncouple these rate order ratios. However, plotting the inverse of the burst timing against the burst size presented four possible noise classes with each class containing a dominant intra-ratio rate contributor to the measured noise. Assessment of the plot identified that (1) all the non-perturbed proteins reside in one identified noise class defined as II.A. (2) Modulation of p53 expression in MCF7 cells, through reducing the proteins degradation rate through the addition of Nutlin, resulted in a rightward shift from II.A into another identified noise class III, which corresponds to a decreased rate of protein degradation. (3) In contrast to this, we see Rubisco expression for healthy cells resides in class II.A and upon heightened degradation rates in senescent cells, a leftward shift occurs into the noise class I, which corresponded to an increased rate of protein degradation. Generally identifying, for the proteins examined in this thesis, that the measured mean protein copy number is representative of the average content per translational burst (i.e. k_2 scales with copy number). While noise generation around this measurement is founded in a relationship between protein and mRNA stability (i.e. k_2/γ_2 and k_2/γ_2 respectively) coupled with cell cycle division events (i.e. extrinsic noise limit).

Obtaining measurements of noise in Rubisco expression from single cell to organelle permitted insight towards noise amplification with respect to the process of cellular

development. We find measurements for total noise (i.e. measurement variability) in Rubisco expression to increase with the complexity of the system measured. Where the complexity, to the best of our knowledge, is evaluated upon the number of noise sources in the total noise measurement. We find that the 1st chloroplast population, composed of low-level extrinsic noise coupled with the intrinsic noise of Rubisco production exhibits a low noise of ($CV^2_P = 0.04$). After the chloroplast division event, identified as the 2nd chloroplast population, partitioning noise is included in the noise measurement ($CV^2_P + CV^2_R = 0.08$). As chloroplast division occurs asynchronously within a single protoplast, these random chloroplast-division events lead to a noise level of ($CV^2_P + CV^2_R + CV^2_E = 0.18$). The summation of these single protoplasts would lead to the highest complexity and exhibits a noise of [$\Sigma (CV^2_E + CV^2_P + CV^2_R) = 0.43$].

8.4 Future Work

The microfluidic affinity capture chip is founded in the principles that accurate quantification of protein content is critical to understanding basic molecular and cellular functions. In hopes that producing an output of absolute protein numbers and noise will have an impact on the parameters of mathematical models, increasing their predictive power. However, we find that protein abundance is only a small measurement of the much larger multi-omic cellular system. Illuminating a need for multiplexed single-cell measurements that this device may not be able to deliver. The chip technology contains practical limits in throughput arising from the number of analysis chambers and the tedious approach of optical trapping for single cell isolation. While the quantitative assay is reliant upon antibodies which hinders the ability to measure different classes of proteins (i.e. membrane-integrated proteins) due to a lack of available antibodies presenting practical limits in multiplexing for whole proteome analysis. While we attempted to address some of those problems in the form of microwell development, to address throughput, or the creation of a secondary standard permitting absolute fluorescent protein quantification in epi-fluorescence microscopy, to allow the detection of proteins that lack available antibodies. It remains evident that additional approaches in multiplexing, temporal measurements, and a reduction in the limit of detection would massively benefit the future work of this device, while opening the possibility of facilitating multi-parameter modelling to enable useful foresight in designing synthetic biological systems.

Multiplexing, through the incorporation of quantitative mRNA measurements, would be highly beneficial as this would allow the uncoupling and determination of the dominant rates within the central dogma of molecular biology, as highlighted in Chapter 7. It would also be beneficial to incorporate quantitative miRNA (microRNA) measurements. As the variability between the relationship of protein and mRNA lifetimes can be affected by the presence of miRNA through inhibiting translation or increasing mRNA degradation rates [57]. Furthermore, the ability to achieve the integration of miRNA detection into the MAC chip microfluidic technology is not a farfetched idea. As recently proved in work presented by Tentori et al. such that microfluidic miRNA detection is accomplished by the integration of hydro-gel posts [255].

Temporal measurements of absolute quantification were originally not possible by means of the microfluidic affinity capture chip. However, through bridging the technologies of this quantitative device with the throughput capabilities of epi-fluorescence microscopy. Future quantitative work of FPF's and FP's may be carried out with temporal resolution. This would guide valuable insight, far greater than the steady-state measurements performed thus far, into noise generation with respect to the random process of cell or organelle divisional events [214, 217].

While continued research into the consequence of these asynchronous divisional events upon noise measurements of photosynthetic proteins would benefit from measurements on the accumulation and replication of chloroplasts (arc) mutants. As these mesophyll cells would, in theory, present less noise than their non-mutant counterparts due to a reduction in the amount of chloroplast divisional events [147]. While maintaining, with respect to leaf size, identical Rubisco and absolute protein contents [257]. This would present beneficial evidence when designing synthetic biological systems for the creation of homogenous mesophyll protoplasts containing chloroplast-derived vaccine antigens or biopharmaceutical proteins.

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