

Imperial College London
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**Computer vision tools to automate the study
of Schlemm's Canal inner wall cell
biomechanics and pore formation**

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Statement of Originality

The work presented in this thesis is the work of the author. All work included in this thesis that is attributable to persons or organizations other than the author has been appropriately referenced or disclosed.

Much of the work presented in this thesis relies on data gathered by persons other than the author. A list explicitly disclosing the origins of the various datasets used in this thesis is included below:

1. Fluorescent microscopy image data of cells from SC67, SC57g, SC70, SC57g, SC63g, SC64g, used during chapters 2 and 3, was gathered by Dr. Sietse Braakman during the work detailed in [1].
2. Video data of hand-drawn fiducial markers, used in section 4.2 for macro-scale strain tracking algorithm validation, was gathered by the author.
3. Microscopy video data of fluorescent particles on micropatterned substrate, used in section 4.2 for micro-scale strain tracking algorithm validation, was gathered by Jaques Bertrand and provided as a personal communication.
4. Fluorescent microscopy image data of cells from SC60 acquired using the modified two-timepoint fluorescent assay, used in section 4.3, was gathered by the author.

Abstract

Glaucoma is the world's leading cause of irreversible blindness and is associated with elevated intraocular pressure, which is often elevated due to an increase in aqueous humor outflow resistance. Aqueous humor outflow resistance is thought to be modulated by Schlemm's canal inner wall pores, which form in response to cellular deformation. In glaucoma, pore density is decreased, suggesting that impaired pore formation may contribute to increased outflow resistance and elevated intraocular pressure in glaucoma. Currently available methods of studying these pores have significant limitations and thus our understanding of Schlemm's canal pores remains rudimentary. This thesis aims to provide tools that address the limitations of existing techniques and to provide new techniques which allow for deeper understanding of Schlemm's canal pores.

The first chapter provides a machine-learning system for the detection of pores in previously obtained image data. The second chapter uses machine learning tools to quantify pore density and size, showing that despite controlling cellular deformation, pore formation is reduced in glaucoma, and that pore size also increases with stretch and is reduced in glaucoma. The third chapter presents an algorithm for tracking the mechanical strain in cells in real time and a modification to an existing method of studying Schlemm's canal pores which allows for the investigation of time dependent phenomena, including preliminary evidence that Schlemm's canal cells retain their pore formation ability in subconfluent culture and that Schlemm's canal pores are transient structures.

Together, the presented methods augment the ability to research Schlemm's canal pores by decreasing research time and allowing for new aspects of Schlemm's canal pores to be explored. The findings presented indicate that despite controlling for cell deformation, pore formation ability is still reduced in glaucoma. This suggests that cell stiffness is not the sole reason for the decreased pore density seen in glaucoma.

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Dedication

To my mother, for seeing greatness in me and encouraging me to reach for it,
To my father, for instilling a fascination with STEM in me from an early age,
To Lexie, for being my guiding star.

‘Wir müssen wissen, wir werden wissen’
David Hilbert (1862-1943)

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Abbreviations Used

AC: anterior chamber

AH: aqueous humor

COP: conventional outflow pathway

GV: giant vacuole

HOG: histogram of oriented gradients

IOP: intraocular pressure

JCT: juxtacanalicular tissue

SC: Schlemm's canal

SEM: scanning electron microscopy

SIFT: scale-invariant feature transform

SNR: signal-to-noise ratio

SURF: speeded-up robust features

SVM: support vector machine

TM: trabecular meshwork

Chapter 1

Introduction and background

1.1 Introduction

Glaucoma is the world's leading cause of irreversible blindness [2]. The majority of glaucoma cases are open angle glaucoma, which affects roughly 60 million people worldwide [3]. In open angle glaucoma, the optic nerve becomes damaged by experiencing deformation, typically due to elevated intraocular pressure (IOP); indeed, elevated IOP is the primary risk factor associated with the disease. This increase in IOP is typically caused by an increase in aqueous humor (AH) outflow resistance in the conventional outflow pathway (COP) [4], but the cause of this increased outflow resistance is still poorly understood.

The COP is a drainage pathway for AH located at the base of the iridocorneal angle. The COP consists of several layers of tissue; the trabecular meshwork (TM), the juxtacanalicular tissue (JCT) and the inner wall of Schlemm's canal (SC). AH flows from the anterior chamber, across these three tissue layers, and into the lumen of SC where it drains from the eye via the episcleral veins. The majority of outflow resistance in the COP is generated in region of the JCT and the SC inner wall [5].

The mechanism that governs outflow resistance generation is not currently known, but it is likely that the SC inner wall plays an important role [6],[7]. The SC inner wall is a continuous

monolayer of endothelial cells. As AH flows from the anterior chamber (AC) out of the eye to the SC lumen, it must cross this endothelium. The direction of AH flow perfuses the SC inner wall in the basal-to-apical direction. This puts large stresses on the cells, causing them to deform, forming balloon-like structures known as giant vacuole (GV)s. When SC cells deform, they form micron-diameter pores, either transcellularly or paracellularly [8], and this behavior is retained in culture. In vivo, these pores provide pathways for AH drainage [9]. Importantly, SC inner wall cells from glaucomatous eyes show reduced pore density [10],[11], making SC inner wall pores a key topic of interest in glaucoma research.

Currently, SC inner wall pores are typically studied using scanning electron microscopy (SEM), due to its high magnification which allows for resolving pores in great detail. However, SEM is very time consuming as an imaging technique, and only images small sections of culture area due to the high magnification.

To address the shortcomings of SEM, a new method of visualizing pores in cultured SC cells [1] was developed by adapting a fluorescent permeability assay [12]. This new fluorescent assay allows for cheap, rapid, low magnification imaging of cultured SC cells while still resolving pore locations and numbers. However, the assay is currently limited in effectiveness by the inability of SC inner wall cells to form tight junctions in culture. This results in image data where pore signals are typically difficult to detect due to bright junctional labelling artefacts, and limits the assay to the detection of transcellular pores only. These issues limit the usefulness of the fluorescent pore assay as a method of studying pores. If these shortcomings can be addressed, the fluorescent pore assay has the potential to greatly improve research throughput on SC inner wall pores, accelerating the study of SC inner wall pore characteristics and mechanisms. It is likely that the techniques used for object detection and classification in the computer vision field may provide the answer to unlocking the potential of the fluorescent pore assay.

In light of this, the overall goals of this thesis are to: **(1)** develop a method of improving the detection of pores in images from the fluorescent pore assay. **(2)** To use this developed method to quantify the differences in pore pore density, size and shape between normal and

glaucomatous SC cells. **(3)** To develop tools and methods that enable more in depth study of SC pores.

1.2 Specific aims

Specific Aim 1: To develop a method of automating the processing of image data obtained from the fluorescent pore assay. The issues with the fluorescent pore assay manifest in the data produced by the assay. Pores present in these data as small, dim, rare blobs of fluorescence. The image data has a very low signal-to-noise ratio (SNR) due to the large, bright staining artefacts corresponding to leaky intercellular junctions. This makes manual processing of the image data infeasible at scale, but a machine learning system could be trained to identify pores in the data.

Specific Aim 2: To process the library of data previously acquired using the fluorescent pore assay. When the fluorescent pore assay was first created, it was used to investigate pore formation in multiple normal and glaucomatous cell lines. However, the majority of this data was left unanalyzed due to the difficulty of manually identifying pores. Using the machine learning system developed for Specific Aim 1, it should be possible to make use of this data and potentially gain greater understanding of SC pores and how they differ in glaucoma.

Specific Aim 3: To develop a method of mapping and tracking the mechanical strain field in cultured cells experiencing deformation. It has been established that SC inner wall cells form pores when they experience mechanical strain. However, it is not currently known how that strain is experienced by the cells. A method of determining the strain field in the cell as it is deformed may provide insight into how the cells transduce deformation into pore formation.

Specific Aim 4: To develop a method of investigating the transient properties of SC inner wall cells. The fluorescent pore assay is a promising technique for increasing the throughput of SC pore research. However, it is currently limited to the detection of pores at a single point in time. If this method is extended to provide time resolution of pore formation by simply using different stains to label pores at different time points, the transient behavior of SC

pores can be investigated, which may offer new insights into how outflow resistance increases in glaucoma.

1.3 Background

1.3.1 Glaucoma

Glaucoma is a condition where the optic nerve becomes damaged, and is the globally leading cause of irreversible blindness [2]. The main risk factor associated with glaucoma is elevated intraocular pressure. This increase in pressure induces deformation of the delicate neural tissues of the eye, which damages the axons of the retinal ganglion cells that form the optic nerve [13].

Open angle glaucoma accounts for the majority of all glaucoma cases [3], affects an estimated 76 million people worldwide, and the number of cases is projected to increase [2]. In open angle glaucoma IOP increases, most commonly due to increased aqueous humor outflow resistance. It is still unknown what causes outflow resistance to increase in open angle glaucoma.

1.3.2 The Anterior Chamber of the Eye and Aqueous Humor Flow

The anterior chamber of the eye is filled with AH. AH provides nourishment to the avascular tissues of the anterior chamber. AH exerts a constant pressure on the cornea. Together with the pressure exerted on the eye by the vitreous humour, these pressures prevent the eye from collapsing on itself.

AH undergoes constant turnover and is produced by the ciliary processes at a fairly constant rate. At steady state the volume of the eye is fixed, so the production rate or "inflow" of AH must be balanced by drainage "outflow". AH drains via two main pathways; the uveoscleral outflow pathway and the COP (see Figure 1.1).

The COP is comprised of several layers of tissue, across which AH flows (see Figure 1.2). From the opening of the pathway, at the angle between the cornea and iris, to the lumen of SC, the tissue layers of the COP are: the uveal meshwork, the corneoscleral meshwork and the JCT (the three of which comprise the TM), and the inner wall endothelium of SC. AH flows across these tissues and into the SC lumen, which connects to the episcleral and conjunctival veins [15].

1.3.3 Schlemm's Canal Inner Wall Function

Due to the direction of AH flow through the COP, SC inner wall endothelial cells experience a basal-to-apical pressure drop, which is relatively unique amongst endothelial cells. This

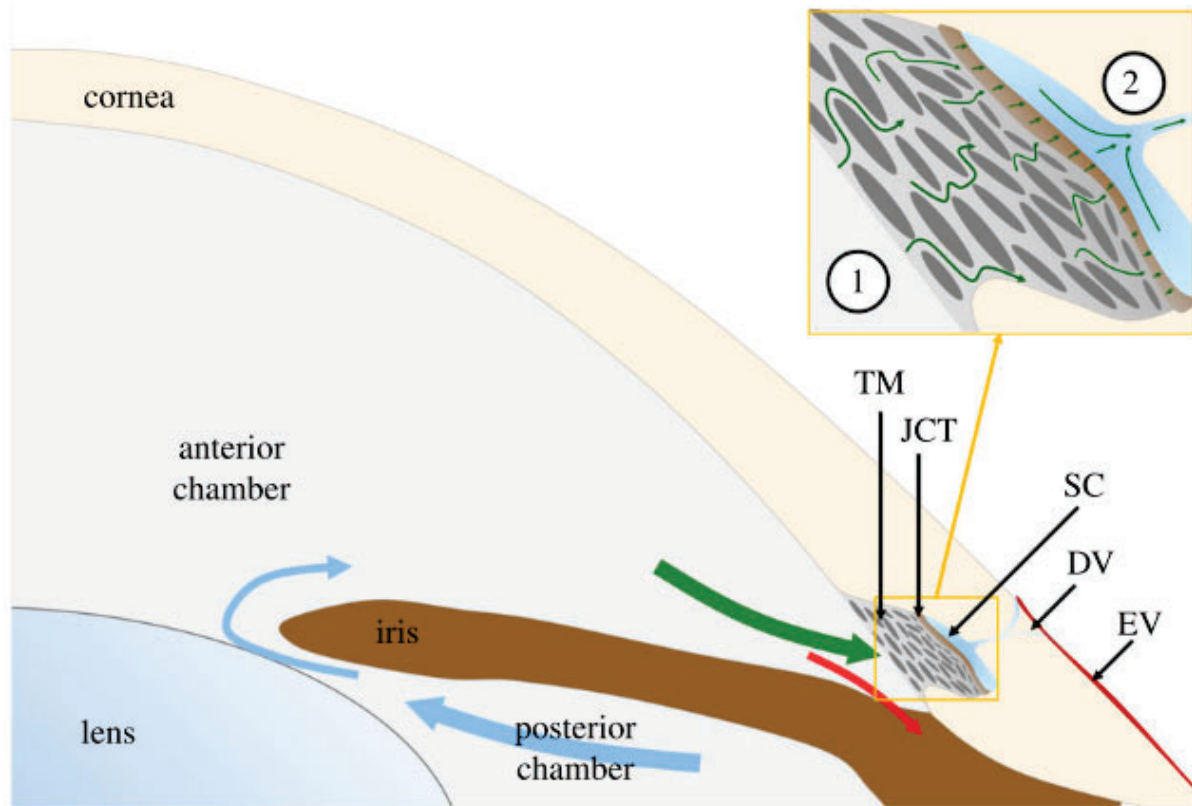


Figure 1.1: Diagram of anterior chamber cross section. Blue arrows indicate AH flow from the ciliary processes into the anterior chamber. Green arrow indicates AH outflow through the COP (green box), across the TM, JCT and SC. (Magnified view) AH flows from the anterior chamber (1) through the COP and into the lumen of SC (2). Red arrow indicates AH outflow via the unconventional outflow pathway (reproduced from [14]).

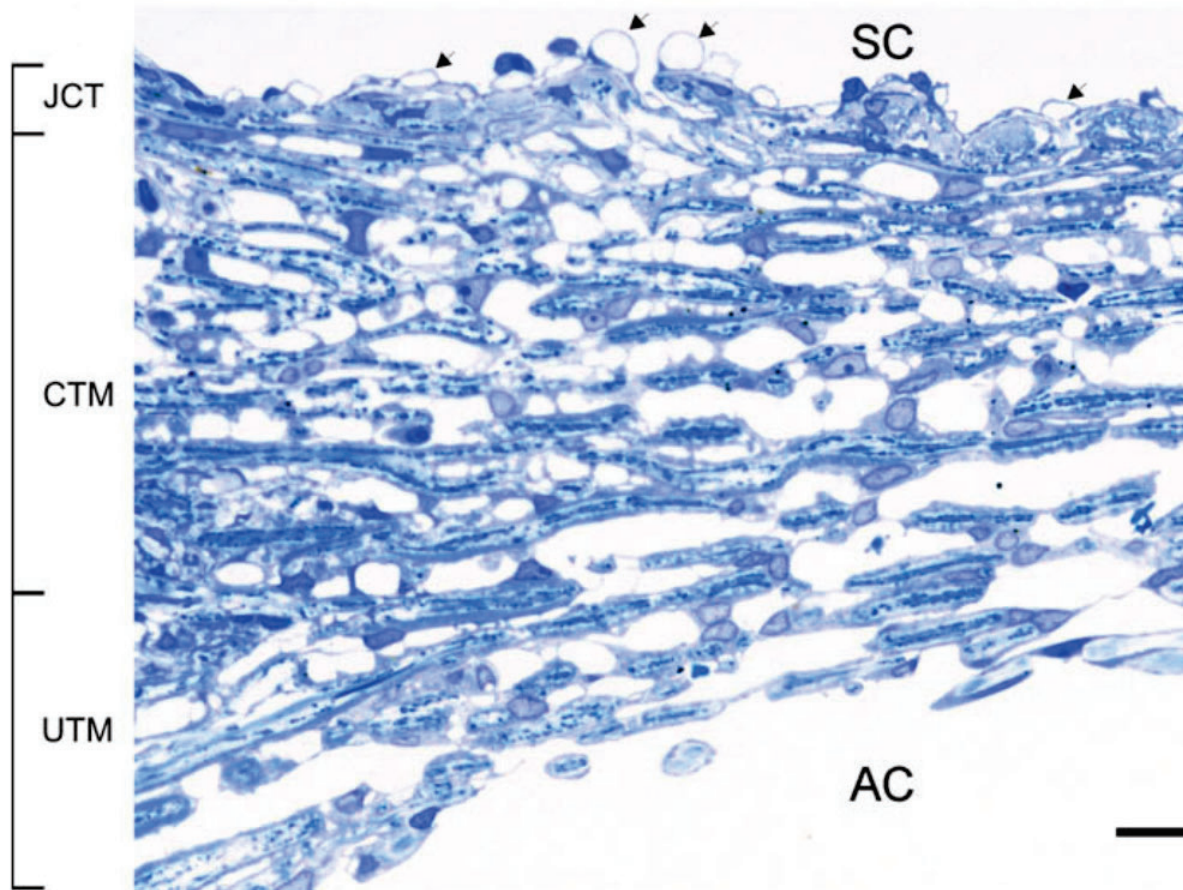


Figure 1.2: Light micrograph of trabecular meshwork section, depicting the distinct layers of the conventional outflow pathway. AH flows across these tissue layers from AC to SC. Arrows indicate giant vacuoles formed by inner wall SC cells. AC: Anterior Chamber; UTM: Uveal Trabecular Meshwork; CTM: Corneoscleral Trabecular Meshwork; JCT: Juxtacanalicular tissue; SC: Schlemm's canal. Scale bar = $5\mu m$ (reproduced from [16]).

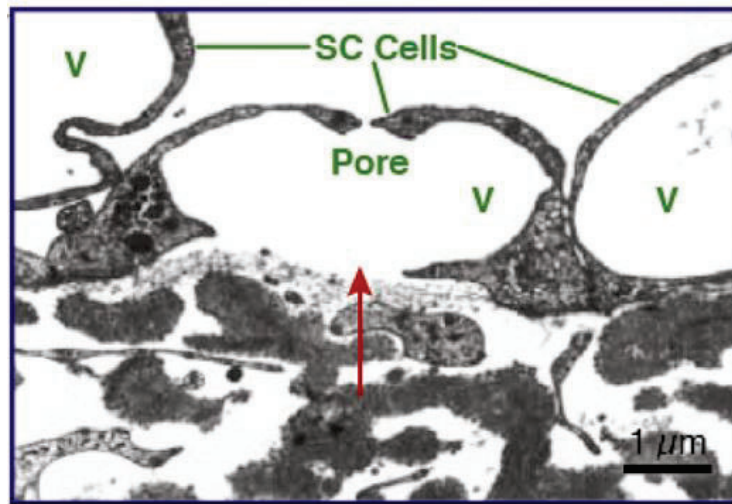


Figure 1.3: Transverse electron micrograph of Schlemm's canal inner wall. Three giant vacuoles are depicted (denoted by green "V"'s), as well as a transcellular pore in the centre vacuole, through which aqueous humour flows. Red arrow indicates aqueous humour flow direction. Cytoplasm appears as dark regions, while intercellular space appears as white (reproduced from [17]).

unusual pressure drop drives three rather unusual cell behaviours: the ballooning of the inner wall into the SC lumen, GV formation and pore formation.

GVs are cellular outpouchings with entirely extracellular cavities that form when the SC inner wall is perfused in the basal-to-apical direction (see Figure 1.3). GV formation places the SC cell under extreme mechanical strains ($\sim 50\%$) [18], indicating that SC cells are uniquely adapted to bear large deformations. Interestingly, GV's collapse very quickly (usually within 3 minutes) when the basal-to-apical pressure drop is removed [19]. This suggests that GV formation sites indicate the presence of large pressure differentials across the SC inner wall, and that their formation and collapse are dynamic processes.

The inner wall of SC is a continuous monolayer containing tight junctions, but AH can flow across it into the SC lumen, due to the formation of transendothelial pores (see Figure 1.3). These pores can form in the junctions between cells (paracellular) or within and across the body of a single SC endothelial cell (transcellular). These pores provide entirely extracellular portals across the SC endothelium for AH to flow through, and are the likely pathway for flow across the inner wall [9]. Pore formation is a mechanosensitive process and can be induced by applying mechanical strain to SC cells [8], or by applying a basal-to-apical pressure drop

across the cell layer [20], although the subcellular mechanisms that govern SC pore formation are currently unknown. Pores tend to colocalize with GVs [21], allowing AH to fill the GV and then exit along the pressure gradient, into the SC lumen through a pore. Little is known about the time-dependent behavior (if any) of SC pores.

1.3.4 Intraocular Pressure and Aqueous Humor Outflow Resistance

The pressure due to the presence of AH is IOP. IOP is described by the Goldmann equation as:

$$IOP = \frac{F_{in} - F_u}{C} + P_v \quad (1.1)$$

Where F_{in} is the rate of AH production, F_u is the outflow through the unconventional outflow pathway, P_v is the pressure in the episcleral veins (where AH drains to) and C is outflow facility. C is most intuitively interpreted as a conductivity to AH outflow. The inverse of C is known as resistance to AH outflow, or “outflow resistance”.

The exact mechanism that governs outflow resistance is not currently well understood.

Maepea and Bill [5] found that in the COP of monkeys, the bulk of outflow resistance is generated within 14 μ m of the SC lumen. Within this distance, three tissues exist; the SC inner wall, the inner wall basement membrane, and the JCT.

The resistance generated by any one of these three tissues is insufficient to explain the high outflow resistance in the region, and the resistances of the three tissues sum to substantially less than the reported resistance values in that area [22]. Ultrastructural studies failed to find an explanation for high outflow resistance generation within the JCT itself [23], and Bill and Svedbergh [6] observed that the high number of pores meant that pores themselves would contribute less than 10% of outflow resistance. However, it was later discovered that the act of fixation causes an increase of pore density [7], suggesting that true SC pore density might be lower than previously reported, thus making SC pores a more important contributor to

outflow resistance generation.

In light of these findings, it is more likely that the bulk of outflow resistance observed is generated by some form of interaction of these tissues. One such model is known as the "funneling model", originally proposed by Johnson et. al [24] and supported in [22]. The funneling model posits that the proximity of the JCT, modeled as a homogeneous isotropic porous material, to the SC inner wall, modeled as an impermeable membrane containing evenly spaced pores, causes flow to be funneled towards pores, increasing the resistance of the JCT over what would be seen if the SC inner wall was not present (see Fig. 1.4). Currently the funnelling model posits that the increase to JCT outflow resistance due to funneling E obeys the following relationship:

$$E = \frac{1}{4naL} \quad (1.2)$$

where n is SC pore density, L is the JCT thickness and a is the radius of the basement membrane discontinuity (originally considered to be the radius of the pore in [24], but later revised to the radius of the GV associated with the pore in [22]) . Using pore radius produced an expected increase of 30 fold, while using GV radius produced a 15 fold increase, both of which are higher than what might be expected from experimental results [25]. This suggests that the relevant discontinuity's radius is larger than that of the GV scale.

1.3.5 Relevance of Schlemm's Canal Biomechanics in Glaucoma

The majority of outflow resistance is generated within the COP [27]. SC pores are the only means by which AH can drain across an otherwise impermeable monolayer. This means that the inner wall of SC likely plays a role in the regulation of outflow resistance.

It has been shown that SC pore density is reduced in glaucomatous SC cells [10],[11].

Glaucomatous SC cells display increased subcortical stiffness compared to non-glaucomatous SC cells, and increased sensitivity to the stiffness of their environment [20], as well as altered

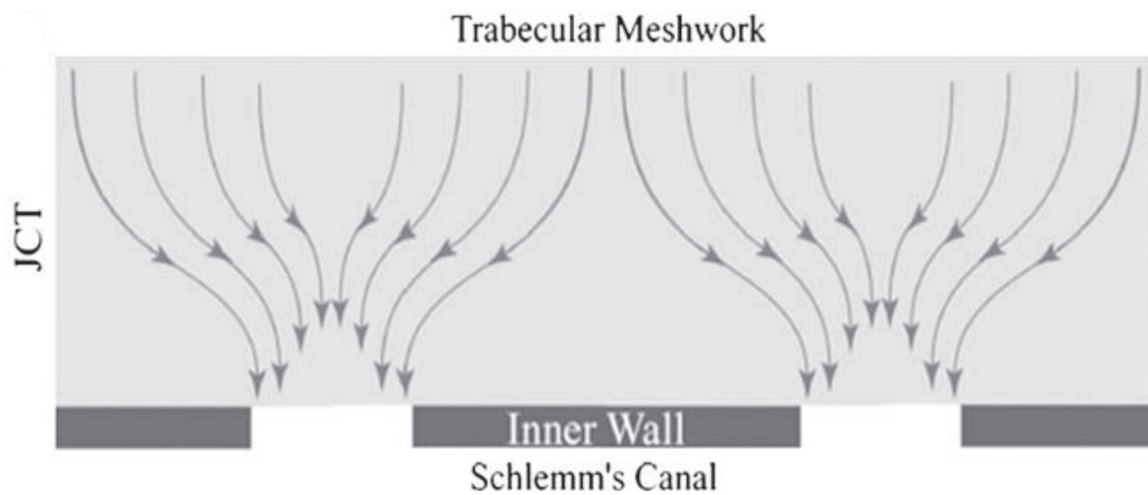


Figure 1.4: Diagram depicting the hypothesized AH flow paths through the JCT according to the funneling model (reproduced from [26]).

gene expression linked to ocular hypertension in mice [28]. Amir et al. found that the inner wall exhibits increased stiffness in glaucomatous eyes, and that this increased stiffness lies immediately below the inner wall surface [29]. W.D. Stamer et al. [30] also presented several studies in support of the notion that SC cell stiffness and AH outflow resistance are linked. Overby et al. [20] demonstrated that these altered biomechanical characteristics impair glaucomatous SC cells' pore forming ability.

The studies mentioned above were conducted under a fixed load, but SC cells have also been studied under fixed mechanical strain, where the deformation itself is applied, rather than the pressure drop which drives the deformation. Using this strain-controlled experimental paradigm, there is limited evidence to suggest that glaucomatous cells may be less able to form pores when stretched [1]. This would suggest that the reduced pore formation ability of glaucomatous SC cells is at least partly independent of cell stiffness, since the stretch experiments conducted in [1] impose a mechanical strain on the cell and thus force an equal deformation on both normal and glaucomatous cells.

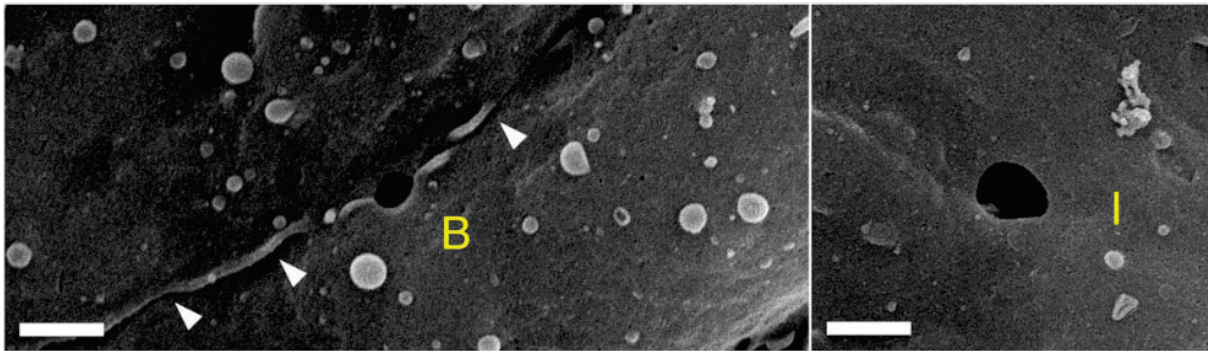


Figure 1.5: Example SEM image of SC inner wall cells forming pores. Left panel depicts a paracellular pore (Indicated by yellow "B"). Border between cells is denoted by white arrow-heads. Right panel depicts a transcellular pore (Indicated by yellow "I"). Scale bars = $1\mu\text{m}$ (reproduced from [8]).

1.3.6 Methods of Visualizing Schlemm's Canal Pores

The gold standard method of visually inspecting SC inner wall pores is currently through the use of SEM (see Fig. 1.5). SEM allows for very high magnification, and thus high detail, imaging of cultured SC inner wall cells. However, SEM is a very time consuming process. Additionally, because of the high magnification, SEM is only feasible for investigating very small areas, meaning the number of SC cells observed in SEM pore assays is low.

SEM studies also have to deal with the artefacts introduced by the fixation process. Fixation is required for SEM imaging, but itself induces pore formation to such a high degree that most or all pores observed via SEM may actually be artefacts [10], which may obscure differences between experimental conditions, hindering the usefulness of SEM as a technique to discern differences in pore formation ability.

Dubrovskiy [12] created a method of visualizing cultured cell monolayer permeability using fluorescent stains (see Figure 1.6). The assay involved culturing a monolayer of cells on a biotinylated gelatin or collagen substrate. Avidin conjugated with FITC (FITC-Avidin) was introduced to the culture medium to begin the staining process. Avidin has a high affinity for the biotin bonded to the substrate, which lies below the culture monolayer. Thus, wherever the FITC-Avidin is able to pass across the monolayer, it binds strongly to the substrate. To remove excess stain that did not cross the monolayer, the culture was rinsed with PBS. To

visualize the results of the permeability assay, the cells were first fixed with formaldehyde, then viewed under a microscope and imaged with a digital imaging system. Because the assay is designed such that FITC-Avidin only remains after washing where there was a gap in the cell layer, the resulting images showed cell bodies as dark areas, and gaps in the monolayer as bright spots.

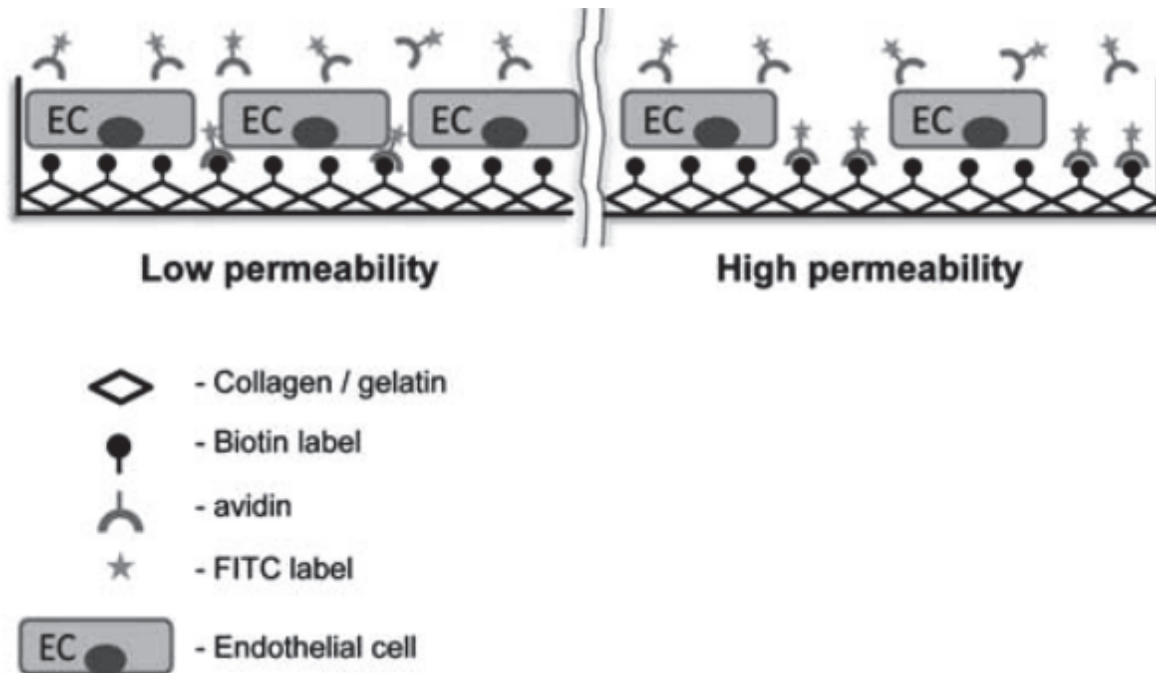


Figure 1.6: Diagram describing how the cell permeability assay functions. Avidin conjugated fluorophores can only cross the confluent culture monolayer where there are permeable areas, irreversibly binding to the substrate beneath the monolayer and thus labelling permeable regions. Reproduced from [12]

This assay was recently adapted and applied to the task of pore detection in SC inner wall cell cultures [1], and appears to be a promising alternative to SEM, as it is cheaper than SEM, allows for investigation of more cell area and avoids the issue of fixation artefacts being indistinguishable from pores. However, because SC cells do not form tight junctions when cultured, images obtained using this method tend to have large artefacts that are far brighter than the pore staining (see Figure 1.7 for example). These artefacts reduce the SNR of the pore signal. The fluorescent assay also reduces pores to largely featureless blobs of fluorescence, making distinguishing pore staining from noise or artefacts difficult and highly subjective. Pores are also rare structures which, when combined with the aforementioned

issues, makes pore identification with this method difficult and time-consuming. Despite this, the possible advantages of the fluorescent assay are attractive enough to warrant its continued use, although a method of mitigating its disadvantages is necessary to fully leverage the method's benefits.

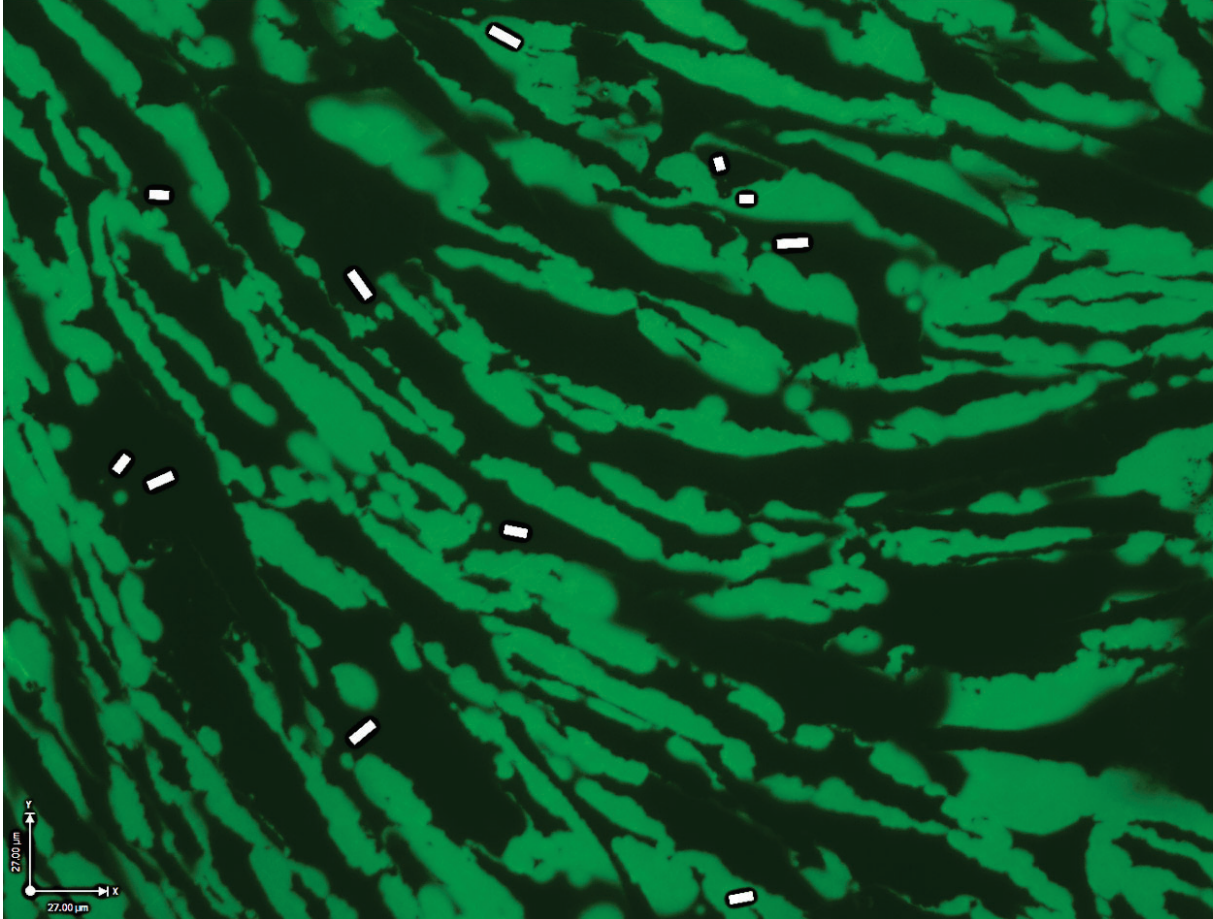


Figure 1.7: Example image from the adaptation of the fluorescent assay described in [12] to SC inner wall cell culture. Brightness and contrast have been adjusted for better visibility. Black areas correspond to cell bodies. Transcellular pore locations are indicated with white rectangles. Other large stained areas indicate leaky intracellular junction locations (original image captured by S. Braakman in [1]).

1.3.7 Computer Vision Approaches to Object Classification in Image Data

Computer vision is the application of machine learning techniques to complete visual tasks. Computer vision is broadly used across multiple industries for a wide variety of tasks, from

detecting humans within scenes [31] to quality assurance on assembly lines [32] to counting colonies in petri dishes [33]. Common computer vision problems include blob detection and object recognition.

In computer vision, a "blob" is typically considered a group of neighbouring pixels that share similar characteristics, such that the group appears uniform. Blob detection is then automated detection of blobs that have differing characteristics than their surroundings, such as color or brightness. Blob detection has many uses, but is very commonly used as a method of detecting interesting areas of images. This particular application of blob detection is known as interest point detection.

Object recognition is the application of algorithms to image data in order to classify the object within the image as one of two or more classes. While object recognition algorithms can vary wildly in terms of specific algorithms used, order of operations and general approach, a typical object recognition system will consist of several identifiable processing steps:

1. Interest point detection
2. Feature extraction
3. Classification

Interest point detection is used when blobs in an image are likely to be the objects of interest within said image that need to be isolated for further study. This approach improves efficiency by scanning the entire image with relatively inexpensive processing techniques to identify these interesting points. In subsequent processing steps, which are typically far more computationally expensive, only the interest points in the image (and their immediate surroundings) are processed, greatly increasing the efficiency of the computer vision system. The benefits of using an interest point detector as a preprocessing tool increase as the rarity of interesting points increases, since more of the image is omitted from further processing.

After interest points have been detected and before the classification of those interesting points occurs, their locations are typically passed to a feature extractor. A feature extractor

is an algorithm which applies a series of operations to an area around an interest point. The result of these operations are feature data. These data encode various properties inherent in the image data but not explicitly described by the raw data, and typically produce a better representation of the contents of an image to machine learning algorithms than the raw data would itself, hence their widespread use in machine learning tasks. Even in computer vision applications where feature data is not explicitly extracted, such as in neural networks, the approach can still be seen, as this is essentially the function of the intermediate layers of typical computer vision neural network architectures. There are myriad feature extraction algorithms, and each has its own merits and disadvantages. Commonly used feature extraction algorithms include scale-invariant feature transform (SIFT) [34], speeded-up robust features (SURF) [35] and histogram of oriented gradients (HOG) [31].

Once feature data is extracted from the image data at each interest point, each feature vector must be classified. The choice of classifier is usually dependent on the specifics of the problem to be solved. In an ideal scenario, the best performing classifier for most problems will usually be a member of the neural network family. However, these models require very large amounts of data to train and can require expensive dedicated hardware and/or large amounts of training time, and so are unsuitable for problems where these resources do not exist or are too costly to procure. Luckily, there are many other classifier architectures, each with advantages and disadvantages. These include support vector machine (SVM) [36], nearest neighbour classifiers [37], and ensemble methods such as random forest [38].

Typically the nature of the problem will influence the selection of classifier for the task. Some problems have datasets where one data class is rare compared to others. This is known as class imbalance, and large class imbalances can significantly reduce the performance of typical classifier architectures. To address problems such as class imbalance, modifications are typically made to existing classifiers so that they are better able to cope with the problem. One such example of a classifier commonly used for handling data with class imbalance is RUSBoost [39].

RUSBoost comprises two techniques: random undersampling of the majority data class and

Adaboost [40]. Randomly undersampling the majority data class reduces dataset size (thereby reducing training time) while essentially redistributing the data to reduce the class imbalance, thus emphasizing the minority class in the data passed to learners¹ compared to the original dataset. Adaboost is a commonly used member of the "boosting" family of techniques, where weak learners are created iteratively to produce an ensemble of weak learners², which are combined to predict the class of unseen examples. Adaboost starts with an equal weighting for each training sample, then updates the weights after a weak learner is created, increasing the weight of misclassified samples and decreasing the weight of correctly classified samples, before training a new weak learner using the newly updated weights. Each weak learner is kept, and once the desired number of iterations is completed, the weak learners are combined as an ensemble to classify new data.

Classification algorithms typically take data as an input, and produce a model of the data as an output. This is commonly referred to as "training a model" in machine learning. This model can then be used to infer things about new data by providing that data as input to the model, which outputs decisions on the class of the data presented as input. To gauge how well a model has learned during training, the model can be shown new data where the correct classifications are known, typically referred to as a "ground truth". The output of the model can then be compared to the ground truth, and its performance can be quantified for comparison against other architectures or previous iterations. This process is known as "testing a model". This process is often cyclical in practice, where a model is trained, tested, and changes are made to the system before repeating the process, aiming to show improvement over the previous model's performance. This optimization process can be done automatically using typical optimization techniques, or manually using the practitioner's experience and intuition (see Figure 1.8).

To evaluate and compare different models, standardized performance metrics are used. In the

¹A "learner" is essentially any machine learning model. However, when generating ensembles of learners, such as in the case of boosting algorithms like Adaboost, learners that are very quick to train and compact to store are typically preferred, such as decision trees.

²A "weak" learner is a learner that performs marginally better than random guessing. These are often truncated forms of normal learners, such as decision stumps, and are faster to train at the expense of worse performance.

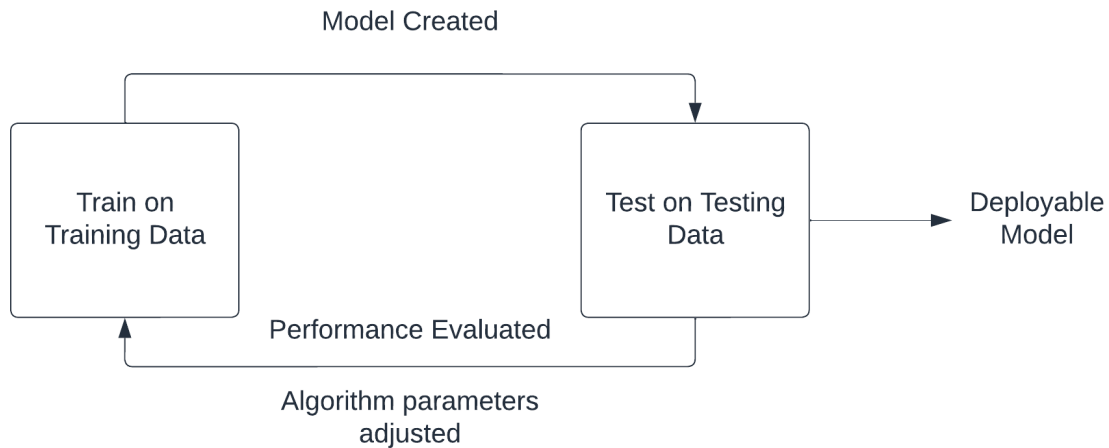


Figure 1.8: Diagram of the iterative process of training and testing machine learning models.

simplest case of binary classification (where the dataset consists of examples of only two classes), each example's prediction can be assigned one of four categories based on what the model predicted for that example and what the ground truth for that example was (see Figure 1.9). If the classes are "positive" and "negative" (as is typically the case when searching an image for a particular object type), then the four categories can be considered as:

1. True Positive (TP). A "positive" example classified as "positive" by the model.
2. False Positive (FP). A "negative" example classified as "positive" by the model. Also known as a Type I error or a "false alarm".
3. True Negative (TN). A "negative" example classified as "negative" by the model.
4. False Negative (FN). A "positive" example classified as "negative" by the model. Also known as Type II error or a "missed detection".

These measures are typically displayed in a tabular form, referred to as a "confusion matrix". All commonly used performance metrics can be defined in terms of these concepts, and extended to multi-class problems. The most commonly used and simplest performance metric is accuracy, defined as:

True Class	+ve	True Positive (TP) Correct classification of +ve class	False Negative (FN) Incorrect classification of +ve class
	-ve	False Positive (FP) Incorrect classification of -ve class	True Negative (TN) Correct classification of -ve class
		+ve	-ve
		Predicted Class	

Figure 1.9: Example confusion matrix. Row labels indicate the ground truth class assigned to each example. Column labels indicate the class assigned to each example by the classifier.

$$Acc = \frac{TP + TN}{TP + FP + TN + FN} \quad (1.3)$$

Which is simply the ratio of correct predictions to the total number of predictions. Alongside, accuracy, the typical performance metrics used are true positive rate (TPR) and false positive rate (FPR), defined as:

$$TPR = \frac{TP}{TP + FN} \quad (1.4)$$

and:

$$FPR = \frac{FP}{FP + TN} \quad (1.5)$$

TPR is the ratio of correctly identified positive examples to the total number of positive examples, which can be interpreted as the classifier's "hit rate". TPR is also commonly referred to as recall. FPR is the ratio of false alarms to total number of negative examples. Typically, models will be optimized to maximize TPR and minimize FPR.

It is important to note that no performance measure is "one size fits all", and that the nature of the data may reduce the usefulness of certain performance measures. For example, the usefulness of FPR decreases as the order of magnitude between FP and TN increases, as is commonly the case in datasets with large class imbalances, resulting in scenarios where a model may produce too many FP s to be useful despite having a low FPR. Because of issues like this, additional performance metrics have been adopted over time. To address the FPR's weakness on imbalanced datasets, precision is often used in its place. Precision is defined as:

$$Precision = \frac{TP}{TP + FP} \quad (1.6)$$

Precision is the ratio of true positives to the total number of positive classifications by the model, which provides a meaningful indication of Type I error rate that is irrespective of class imbalance. Recall and precision are typically considered gold standards as base performance metrics in machine learning applications, and models will typically be optimized to maximize both these measures.

If no particular importance is placed on recall or precision over the other, then the F1 score of the model can be optimized instead, which is defined as:

$$F1 = \frac{TP}{TP + \frac{1}{2}(FP + FN)} \quad (1.7)$$

Which is simply the harmonic mean of recall and precision. F1 score provides a method of comparing models with a single measure that is robust to common issues such as class imbalance, and is therefore often reported in machine learning literature.

1.3.8 Summary

1. SC cells likely play a key role in modulating outflow resistance, and SC pore formation is likely a key part of how outflow resistance is modulated.

2. SC pore formation is triggered by mechanically deforming SC cells, although little is known of the mechanotransduction mechanisms involved or the transient behavior of SC pores.
3. SC pore density is reduced in glaucoma, both due to increased subcortical stiffness and impaired pore formation ability, making SC pore formation a key topic of study in glaucoma research.
4. The current standard for studying SC pores is SEM, which is time consuming and low-throughput.
5. A fluorescent pore assay has shown promise as a successor technique to SEM, and has the potential to enable high-throughput study of SC pore formation.
6. The analysis of image data obtained from the fluorescent pore assay is difficult due to the rarity of pores and the artefacts in the images due to leaky junctional labelling.
7. Computer vision is a field of machine learning that commonly deals with the problem of recognizing objects within image data, and may provide methods to improve pore identification in the fluorescent pore assay, as we will discuss in the next chapter.

Chapter 2

A Machine Learning System for Identifying Transcellular Pores in Cultured Schlemm's Canal Inner Wall Cells

2.1 Introduction

To drain from the eye, AH passes through micron-sized pores in the inner wall endothelium of SC [9]. SC inner wall pore density is reduced in glaucoma [10], which may contribute to increased AH outflow resistance and elevated IOP. The reduced pore formation ability seen in glaucomatous SC cells is reproduced in cultured SC cells exposed to basal-to-apical directed pressure gradients [20], allowing for the study of this phenotype in vitro.

Previous studies [8] relied on SEM, which is labour intensive and time consuming. To enable cost effective, high throughput study of pore density in cultured SC cells, Sietse Braakman (referred to as STB from here onwards) previously developed a fluorescent assay to label pores in SC cell monolayers [1]. The cells are cultured as a confluent monolayer in an

equibiaxial cell stretcher [41], aiming to replicate the mechanical strain experienced by SC cells in vivo during the process of GV formation and inner wall ballooning. SC cells are cultured on the stretchable membrane of a cell stretcher. The membrane of the cell stretcher is covalently coated with a layer of biotin. When the stretcher is actuated, the cells are stretched, which triggers pore formation. While stretched, the cell layer is exposed to a fluorescent avidin stain. This stain crosses the cell monolayer at locations of high permeability, such as pores in the monolayer, and binds irreversibly with the biotin-coated substrate. This creates fluorescent “hotspots” on the culture substrate that can be imaged with fluorescence microscopy (see Figure 2.1). In this work, we refer to cultured SC endothelial cells from different donors as cell “lines”, rather than the default terminology of cell strain, since the cells cultured are primary cells. This is done to avoid confusion with the mechanical strain applied to the cells during the experiments.

This fluorescent pore assay presents several advantages over the current gold standard technique for SC inner wall pore studies, SEM. The time required to conduct a fluorescent pore assay is a fraction of what is required for SEM studies. This makes the fluorescent pore assay much more scalable, allowing for quick testing of multiple experimental conditions with little additional costs per additional experiment. The fluorescent pore assay also has the advantage of labelling pores before fixation, thereby avoiding the fixative-induced increase in pore density that has been shown to occur in post-mortem human donor eyes [7]. This also means that the fluorescent pore assay should provide a better picture of the pore density solely due to experimental conditions, since the artifacts associated with SEM studies may account for most or all observed pores. Finally, the fluorescent pore assay can be performed at much lower magnification than SEM, meaning a much larger cell area can be examined with this technique.

The fluorescent pore assay produces high volumes of image data that must be examined by a trained human observer to identify individual pores (see Figure 2.1 for example). Because cultured SC inner wall cells form immature intercellular junctions, pores are often difficult to discern from noisy junctional labelling, and only transcellular pores are detectable. SC inner wall pores themselves are also relatively sparse, typically representing roughly 0.02% or less of

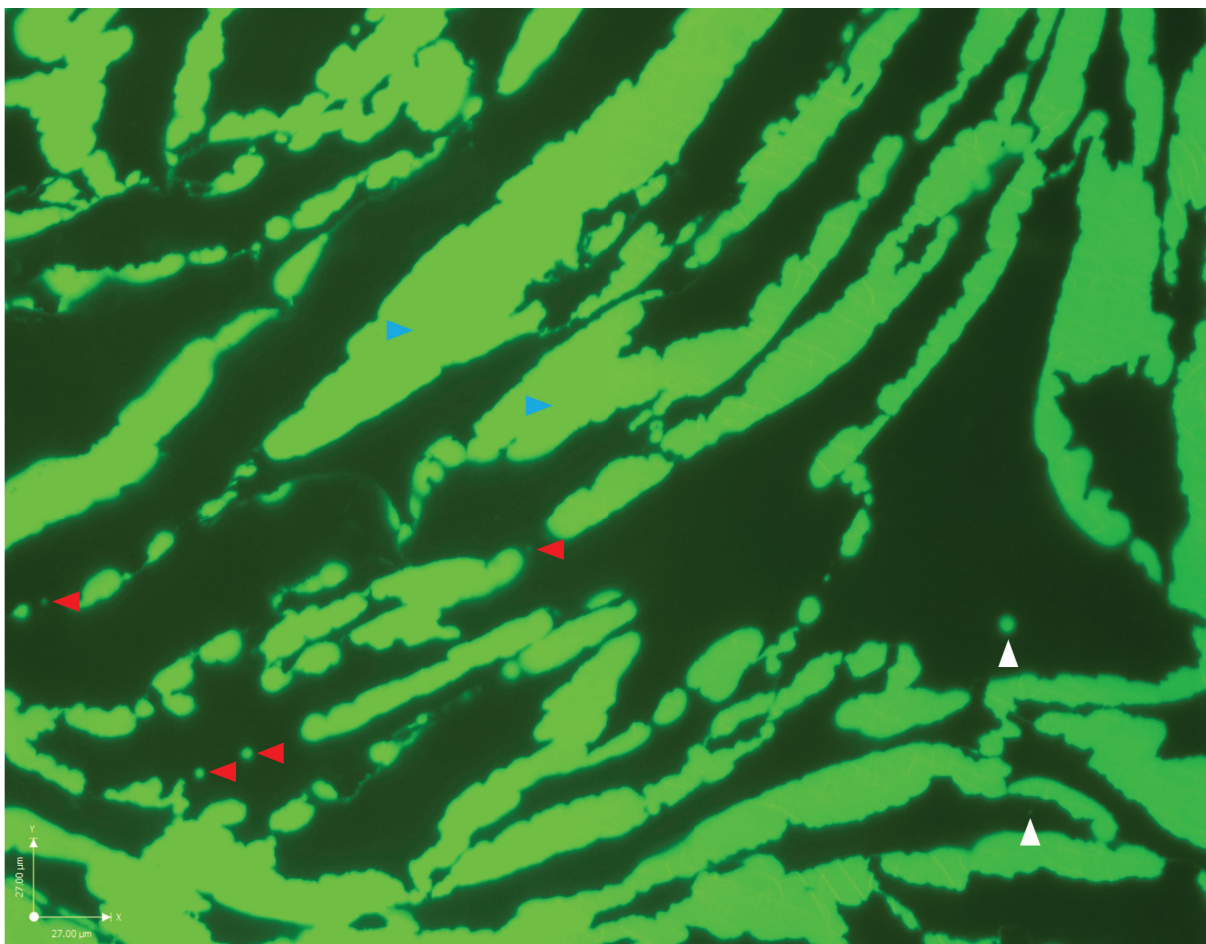


Figure 2.1: Example of image data obtained from the fluorescent assay. White arrowheads indicate two likely pore locations, providing examples of the range of possible sizes and brightnesses for pores in these data. Blue and red arrowheads indicate examples of artefactual staining due to leaky intracellular junctions. Red arrowheads indicate example non-pore objects, illustrating the difficulties associated with automated pore identification in these data. These examples appear to lie on cell borders and are likely staining due to leaky cell junctions, and are thus rejected as transcellular pores. Scalebars (lower left) = $27\mu\text{m}$.

pixels in a given image. These difficulties make manual pore identification and labelling time consuming, labor-intensive and sensitive to sources of variation, such as the marker's interpretation of identification criteria and the equipment used for displaying the images.

These shortcomings decrease the throughput of the fluorescent assay, but if these issues can be overcome, the advantages of the quick turnaround time and high data volume of the assay can be fully utilized. In an attempt to address these shortcomings and fully unlock the potential of the assay, we sought to develop an objective, reproducible and time efficient system for processing the data using machine learning.

SC inner wall pores are rare structures, and transcellular pores represent the minority of the SC pore population which typically present with densities of roughly 40 to 100 pores per mm^2 [8]. This rarity translates to the image data obtained via the fluorescent assay, where an image typically contains between 0 and 15 pores. Because these pores are very small, on the order of 50 pixels in area, and so rare in the raw image data, there is a large inherent class imbalance present in the dataset, since the vast majority of image pixels are non-pore pixels (roughly 1:3500 pore:non-pore pixels¹). This class imbalance is critical, as it means that a typically acceptable false positive rate can result in an unusable model due to the large number of negative examples. Imbalanced data can also lead to misleading performance measures if not accounted for, so the selection of appropriate performance metrics becomes critical. To combat the large class imbalance inherent in the dataset, we opted to use RUSBoost [39], a commonly used boosted ensemble classifier, as our classification algorithm, since it was specifically designed to perform well on imbalanced data.

Overview of model development process in this project

We used the existing raw fluorescent assay image data acquired from previous experiments by STB to train and test our system (see Figure 2.2). We started with the pores identified by STB in [1], making a dataset referred to as "Digital STB" or "DS". STB gathered image data for six cell lines (three normal, three glaucomatous) but only examined the data from two cell lines (one normal, one glaucomatous) due to the limitations mentioned above. Using all the pores identified by STB, we trained and tested a RUSBoost model. When we tested the DS RUSBoost model, we found performance to be subpar, and suspected the issue might be due to errors in data labelling. Thus, we manually verified each pore identified by STB against a set of criteria (identified as part of the work and presented below), to ensure all examples were properly labelled, creating a new dataset referred to as "Digital STB Plus" or "DS+" and training a new RUSBoost model on this dataset. The DS+ RUSBoost model showed improved performance, but was still insufficient. In an attempt to improve performance further, we added data from the remaining four cell lines (two normal, two glaucomatous). To

¹ Average pores per image is roughly 8, at 50 pixels in area on average. Each image contains 1344×1024 pixels and pixel classification is binary (pore or non-pore). This gives a pixel class per image ratio of 400 : 1,375,856 or 1 : 3439 pore:non-pore pixels

do this, we manually identified pores in a random subset of the available images and added it to the manually verified dataset, resulting in another new dataset referred to as "All6". We then trained another RUSBoost model using this dataset. The All6 RUSBoost model showed further improved performance, but still not to satisfactory levels. We finally augmented the feature data with explicit shape measures (area, eccentricity, etc.) to attempt to better distinguish pores from non-pore structures, creating a dataset referred to as "Augmented All6" or "AugAll6", and trained a new RUSBoost model (AugAll6 RUSBoost), which resulted in acceptable performance (see Table 2.1 for summary).

Dataset	SC67	SC57g	SC65	SC70	SC63g	SC64g	Manual Review	Augmented
DS	✓	✓	×	×	×	×	×	×
DS+	✓	✓	×	×	×	×	✓	×
All6	✓	✓	✓	✓	✓	✓	✓	×
AugAll6	✓	✓	✓	✓	✓	✓	✓	✓

Table 2.1: Cell lines included in and additional data handling applied to each dataset.

To investigate whether other models might perform better than the AugAll6 RUSBoost model we trained, we also trained and tested several other classification algorithms on the AugAll6 dataset, including SVMs, decision trees and boosted algorithms. Neural networks were not used because they require larger amounts of data than are available for this study, and require significant resources and time to train. The top three performing models were selected based on cross validation F1 score on the pore class. These models were then applied to an unseen manually validated test set to assess generalized model performance. Finally, the models were applied to "real-world" datasets, representing the full class imbalance inherent in real SC pore data. This "real-world" case includes the pore examples in the manually validated test dataset, as well as more difficult "borderline" pore examples, and the full set of negative examples present in the images, to provide a test case under a realistic class imbalance. Real world performance metrics were calculated and used as criteria for final model selection.

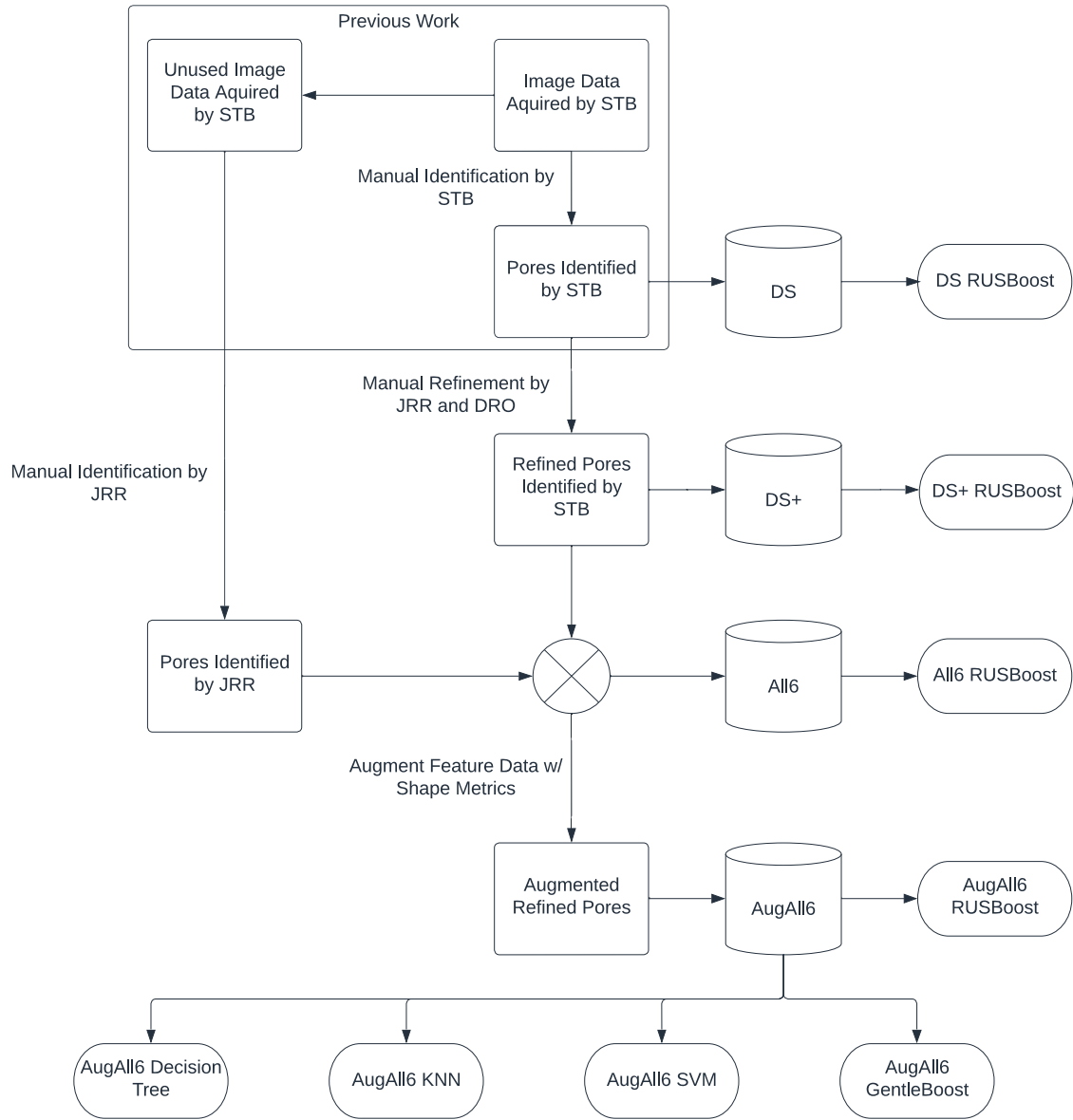


Figure 2.2: Flowchart depicting workflow in developing the pore detector model. Cylinders indicate HOG feature datasets. Ellipses indicate trained classification models. All feature datasets were extracted by applying a custom interest point detector and subsequently applying HOG feature extraction to interest points.

2.2 Relevant Background

This section recounts the experimental work done by STB to obtain the data that is analyzed in this chapter. The work was previously published in [1], but is summarized here for the sake of completeness and necessary background in later sections.

2.2.1 Experimental Design

The experiments involved applying mechanical strain to cultured cells by stretching the culture substrate. To ensure the mechanical strain was transmitted to the cells through the substrate, strong cell adhesion was required. Because the substrate needed to be stretchable and optically clear, PDMS was used, which has poor cell attachment. To address this, the PDMS substrates were covalently coated with gelatin using a glutaraldehyde crosslinker. The gelatin was first covalently bonded to biotin, as this was required for the permeability assay to function by providing binding sites for the avidin conjugated fluorescent tracer.

To perform the stretch experiments, cloning grids were applied to the stretchable substrate to form experimental wells and were covalently coated with biotinylated or non-biotinylated gelatin. Biotinylated wells were used as experimental wells (with cells) and positive control wells (without cells), while non-biotinylated wells seeded with cells were used as negative controls.

Cells were seeded in the experimental and negative control wells and cultured for 4 to 5 days to confluence. Once confluent, the cloning grids were removed and the stretchers applied equibiaxial strain of 0%, 10% or 20% to the substrate for 5 minutes. The cells were exposed to the FITC-Avidin stain during the last 3 minutes of this 5 minute period.

2.2.2 Biotinylation of Gelatin

To biotinylate the gelatin, the following protocol was used:

1. Dissolve gelatin from porcine skin (G2500, Sigma-Aldrich, UK) at 10mg/mL in 0.1M NaHCO₃ at pH 8.3 for 1 h at 37° C
2. Centrifuge dissolved gelatin solution at 2000× g for 20 minutes and collect supernatant
3. Dissolve biotinylation reagent (EZ-link NHS-LC-LC-Biotin, 21343, ThermoScientific, UK) in dimethyl sulfoxide (DMSO, D8418, Sigma Aldrich) at 5.7mg/mL
4. Mix biotinylation reagent solution and gelatin solution at 9:1 at room temperature for 1 hour to allow biotinylation to occur
5. Aliquot biotinylated solution and freeze at -20C for storage until needed

2.2.3 Schlemm's Canal Cell Isolation and Culture

The SC endothelial cells used in the study were isolated from human donor eyes with Stamer et al.'s cannulation method [42] and verified as endothelial cells by measuring expression of VE-Cadherin and Fibulin-2 [43]. All SC endothelial cells used in the stretch experiments were used at passages between three and six.

Cells were cultured in an incubator at 37C in 5% CO₂ at 100% humidity in media comprised of the following:

- 1 g/L glucose Dulbecco's Modified Eagle's Medium with L-glutamine and 25mM HEPES (DMEM, Gibco 22320, Life Technologies, UK)
- 10% Fetal Bovine Serum (FBS, Hyclone SH30070.03, Thermoscientific, UK)
- 1% Penicillin/Streptomycin (P4333, Sigma Aldrich, UK)

Cells were passaged with 0.25% trypsin-EDTA (T4049, Sigma Aldrich, UK) before reaching confluence.

2.2.4 Equibiaxial Cell Stretching Device

The cells were stretched using an equibiaxial cell stretching device, manufactured according to the design established by Lee et al [41]. The stretcher design is a set of three concentric rings; the outermost ring, or "screwtop", the intermediate ring, or "membrane holder", and the inner ring, or "indenter ring". The screw-top, and the membrane holder are threaded, so that twisting the screw-top pulls the membrane holder up, towards the screw-top. The "membrane holder" clamps the stretcher membrane, a thin sheet of PDMS where cells are seeded, into the system and holds it in place with an o-ring as the indenter pushes down on it.

When the threads are engaged but the system is at zero turns, the innermost ring, or indenter, sits flush against the inside face of the screw-top's lip, and flush with the bottom face of the membrane holder, contacting but not deforming the stretcher membrane. As the screw-top is turned, the membrane holder rises, pulling the membrane against the indenter ring, applying an equibiaxial strain to the membrane (see Figure 2.3). A pin inserted into a slot between the indenter and membrane holder ensures the indenter does not rotate relative to the membrane holder, thereby ensuring the membrane does not experience shear stress, enforcing the equibiaxial condition.

Silicone lubricant is applied to the indenter ring to ensure low friction contact between the indenter ring and the stretcher membrane, ensuring the membrane slides smoothly and uniformly over the indenter ring and thus that the strain field is applied uniformly across the stretcher membrane.

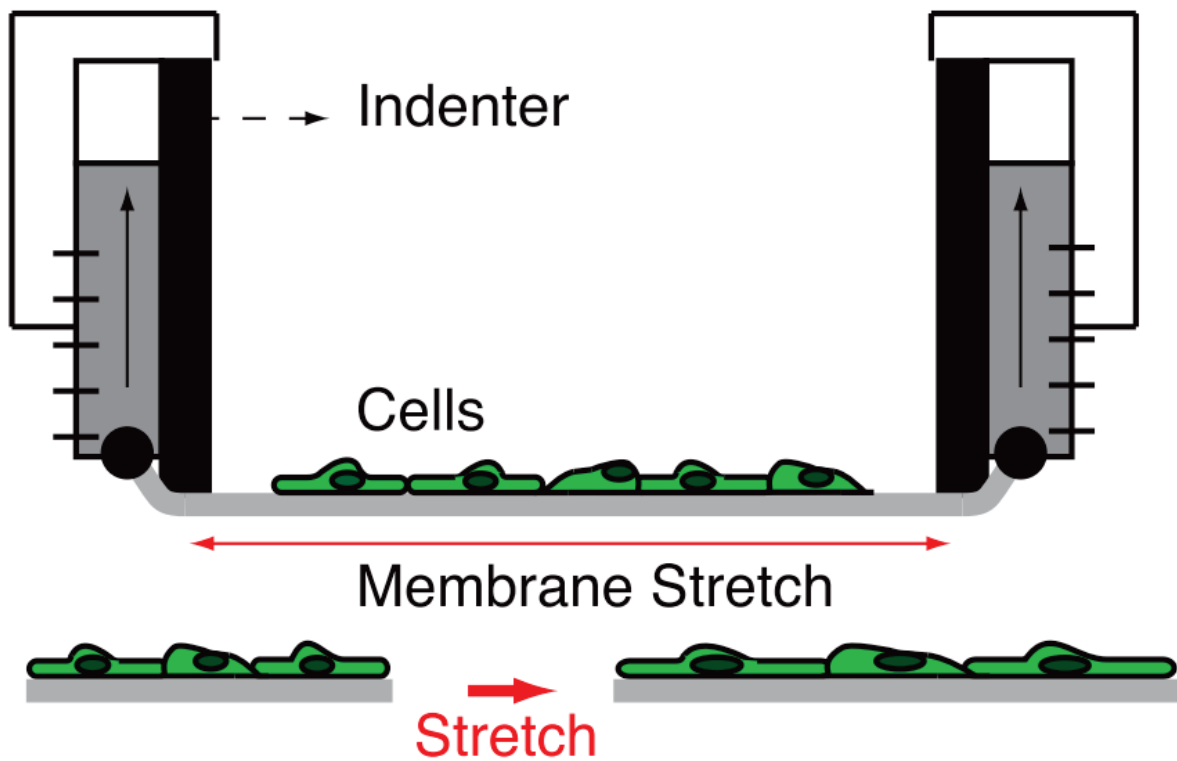


Figure 2.3: Diagram describing the function of the cell stretcher. As the outer ring is rotated, the indenter is pressed into the stretcher membrane, deforming it. This deformation is transferred to the cells attached to the membrane. Reproduced from [1].

2.2.5 Stretcher Membrane Coating

Once the gelatin was biotinylated, it was covalently bound to the stretcher membranes to provide a surface that cells will attach to, and to ensure that strain is transmitted from the stretched PDMS directly to the cells. The biotinylated gelatin stock was defrosted in a water bath and incubated overnight on a layer of chloroform (roughly 10% the volume of gelatin solution) to sterilize the solution. To coat the PDMS membrane with biotinylated gelatin, it was first plasma oxidized for 90 seconds at 0.7mbar (70Pa) and 70 W (Plasma Prep 5, GaLa Instrumente, Germany). The membrane was then submerged in 10% APTES in ethanol for 45 minutes at 55C on a hot plate. The membrane was then washed twice with DPBS and submerged in a solution of freshly prepared 3% glutaraldehyde in DPBS for 20 minutes at room temperature. The membranes were then washed twice again with DPBS. The membrane was then affixed to the cell stretching devices and an autoclaved 4x4 cloning ring grid was then applied to the membrane with autoclaved silicone grease as a sealant to form discrete wells. The sterilized biotinylated gelatin solution, diluted to 0.25mg/mL in DPBS, was applied to eleven of the 16 resulting wells, while the remaining five wells and the area outside the cloning ring grid were coated with unbiotinylated gelatin. The coated wells were left to incubate overnight at 37C in 5% CO₂. Before use, the wells were washed thoroughly with DPBS to remove unbonded gelatin solution.

2.2.6 Stretch Experiments

Three membranes were prepared simultaneously, as described above. Six of the wells treated with biotinylated gelatin and five wells treated with non-biotinylated gelatin were seeded with SC cells at 24,000 cells/mm², forming the experimental wells and negative controls. The resulting cell layers are referred to as specimens. The remaining five biotinylated wells did not receive any cells to function as a positive control. The cloning ring grid was removed six hours after seeding the cells.

When the cells appeared confluent (after 4 to 5 days of culture), strain was applied to the

three stretchers simultaneously. One membrane was left unstretched (0% strain), one was stretched to 10% strain, and one was stretched to 20% strain. The following protocol was followed to perform the pore assay, taking the onset of strain as $t = 0$ s, using solutions warmed to 37C:

1. at $t = 90$ s, aspirate media
2. at $t = 120$ s, expose cells to FITC-avidin solution (diluted 1:100 in DPBS)
3. at $t = 270$ s, aspirate FITC-avidin solution
4. at $t = 300$ s, wash cells with DPBS containing Mg^{2+} and Ca^{2+}
5. at $t = 330$ s, wash cells with DPBS containing Mg^{2+} and Ca^{2+}
6. at $t = 360$ s, fix cells in ultrapure formaldehyde (F017, TAAB, UK) diluted to 4% in DPBS for one hour at room temperature

2.2.7 Image Preprocessing for Pore Identification

To facilitate manual identification of pores, the raw image data were first preprocessed according to the following techniques (see Figure 2.4 for example). Images were first converted from RGB to greyscale. Corrections for uneven illumination were then performed, and contrast was enhanced. Otsu thresholding was then applied to obtain a binary image where values above the threshold were labelled as “on”. Connected components labelling was then performed on the binary image using 4 connectivity to obtain a segmented image. Connected components were pruned with an area threshold. This area threshold was set at 100 square microns. This value was chosen as much larger than typical pore area (1 square micron) to account for the lateral diffusion of FITC-avidin under the cell after passing through the pore. This resulted in a reduced number of objects to manually review by effectively eliminating the fluorescent signal from the leaky cell junctions.

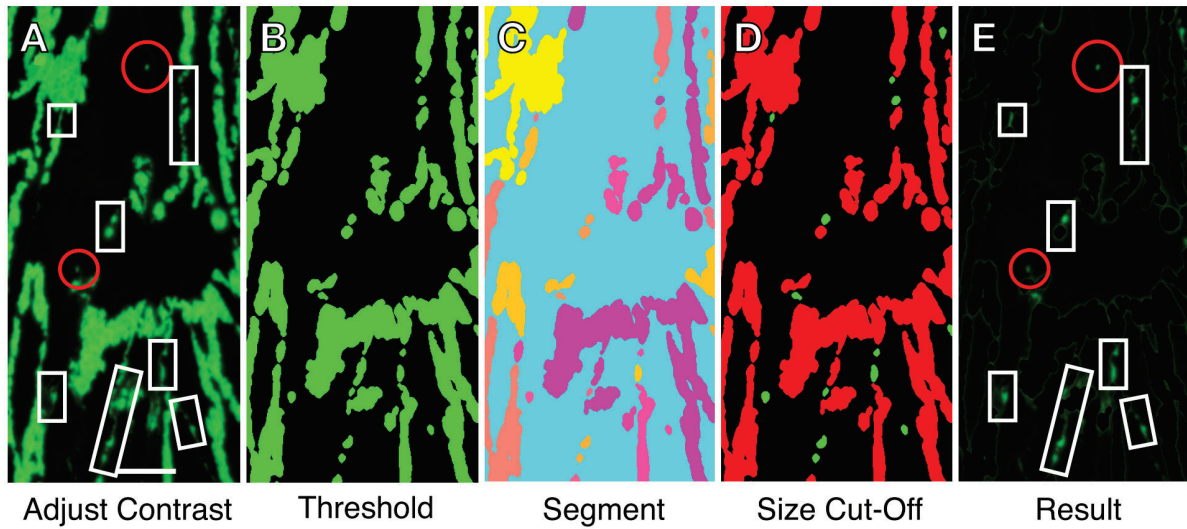


Figure 2.4: Example results from each step of STB's preprocessing algorithm. Red circles indicate putative pores. White rectangles non-pore artefacts missed by the preprocessing algorithm. A) Image after adjusting for uneven illumination and enhancing contrast. B) Results of thresholding A. C) Results of performing connected components labelling on B. D) Results after applying a size cut-off to objects in C. Red objects are removed in the next step, while green objects are kept. E) Result of applying the size cut-off from D to A. Reproduced from [1].

2.2.8 Pore Identification and Counting

Cell Line	Strain	Pore Count	Image #	Pores per Image	Pore Density (mm^{-2})
SC67	0	225	40	5.63	41.45
	10	553	56	9.88	72.77
	20	1044	82	12.73	93.83
SC57g	0	184	29	6.34	46.76
	10	240	36	6.67	49.13
	20	263	30	8.77	64.61

Table 2.2: Pore counts obtained by STB via manual image inspection.

In all, STB identified pores in 287 images, each covering 0.14mm^2 , from one normal (SC67) and one glaucomatous (SG57g) cell line at 0%, 10% and 20% strain (see Table 2.2). STB indicated pore locations by manually drawing indicators on the raw images. This process resulted in the identification of 2,509 candidate pores (see Figure 2.5). Results were normalized by image area examined to give pore densities. Pore density is a metric of how many pores can be expected within a given area, and is typically presented in units of mm^{-2} . Because the leaky intercellular junctions between SC cells produced staining artefacts that

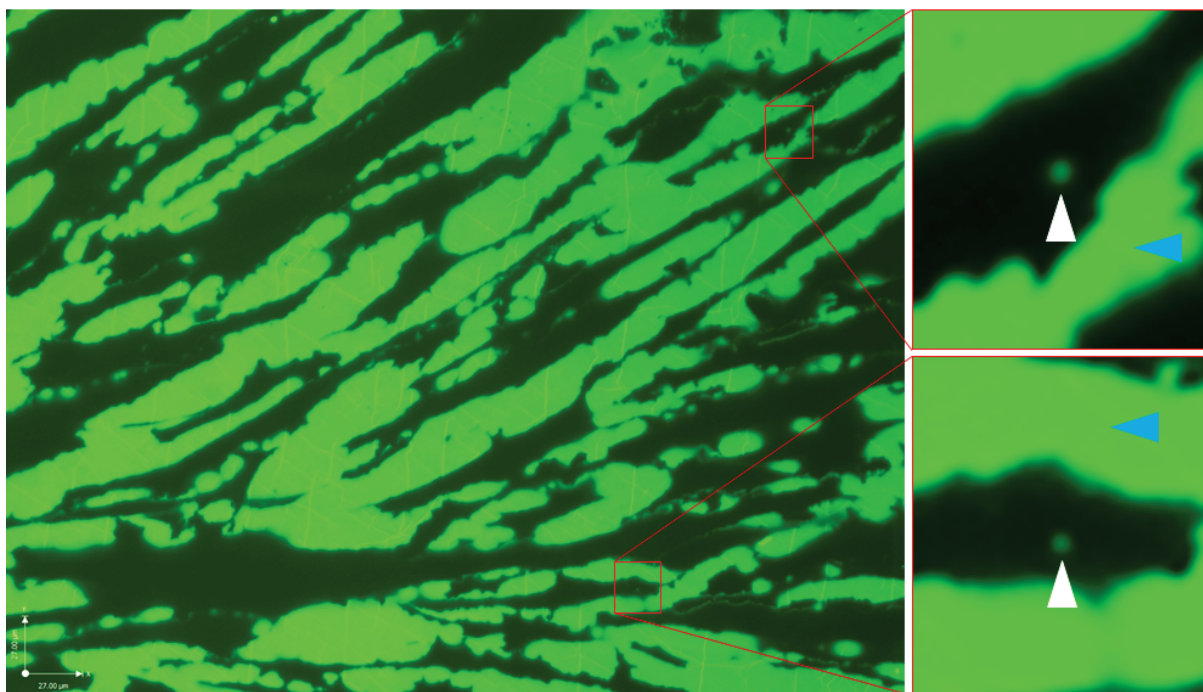


Figure 2.5: Example pores identifiable in the image data produced by the fluorescent assay. Right panels are zoomed in views of sections of the left panel, displaying likely transcellular pores. Contrast, brightness and sharpness have been adjusted for ease of viewing. White arrowheads indicate transcellular pores. Blue arrowheads indicate leaky intracellular junction staining, obscuring any possible paracellular pores and overpowering the signal of transcellular pores, making pore identification more difficult.

made the identification of paracellular pores impossible, only transcellular pores were identified in these data.

2.3 Methods

The following sections detail the work done by the author (denoted as JRR), unless otherwise explicitly noted.

2.3.1 Manual Verification of Previously Identified Pores

As a method of quality control on the data to be used for training and testing the machine learning models, JRR and DRO (Prof. Darryl Overby) reviewed all candidate pores identified

by STB. Based on this review, JRR and DRO designed a set of criteria that define an acceptable transcellular pore (see Figure 2.6 for examples). These criteria are:

- Pore brightness must be similar to that of the border signals in the immediate region and without dark internal regions.
- Pores must be circular in overall shape with a convex hull.
- Pores must have clearly defined and well contrasted borders and must be distinct from cell borders.
- Pores must be larger than 4-8 pixels ($0.36\text{-}0.72\ \mu\text{m}^2$) in area.
- Pores should be manually detected in a consistent colorspace, ideally using a green stain.

We did not define a required minimum brightness for pores. Instead, we stipulated that putative pores must be brightly stained relative to surrounding staining patterns. This helped to account for inconsistencies in brightness (such as that seen on the edges of image tiles). Dim objects relative to the brightness of nearby well labelled sections (typically neighbouring junctional staining) were generally rejected. This was done because the brightness of the noise floor was variable enough to reach the levels of otherwise pore-like objects. Thus, if a putative pore's brightness was similar to the noise floor it was rejected (see Figure 2.6, bottom right panel for example).

High circularity was preferred for pores. Pores typically appeared circular or slightly elliptical in SEM data, and thus we would expect pores to appear similarly in fluorescent assay data. Objects that were highly elliptical tended to be border staining artefacts, further motivating the decision to reject highly elongated putative pores (see Figure 2.6, top right panel for example).

Putative pores were not accepted if they had concavities in their border. While the cellular processes bridging pores sometimes seen in SEM pore images could account for non-circular pore objects in the fluorescent images, this was judged unlikely due to the diffusivity and long

exposure time of the stain. Thus, objects with indentations in their border were rejected. Multi-lobed pores have not been observed thus far, and so multi-lobed objects (objects with dimples in the border) were also rejected.

Pores were required to have well contrasted borders. Some putative pores met the above criteria, but appeared connected to other objects. When connected to junctional labelling artefacts, it was not possible to rule out the possibility that the putative pore was formed by diffusion of stain from the border under the cell. Additionally, because only transcellular pores were considered, putative pores were required to be fully distinguishable from any neighboring junctional labelling artefacts. Thus, any putative pores that appeared to intersect or be associated with junctional labelling were rejected (see Figure 2.6, right panels for example).

Pore clusters were observed in SEM pore images. Clusters of discrete pores were also observed in the fluorescent assay data, but in some cases multi-lobed structures that could be multiple pore stainings bleeding together were also seen. These structures were counted as pores only if they met the above criteria. Such objects still had clear intensity peaks at each distinct pore location in the cluster, and troughs of lower intensity roughly equidistant between the peaks.

We did not impose an upper limit on the pore size. In SEM pore analysis, an upper limit on pore size of 4 to 5 μm in diameter was set. Because it is unknown how much the stain is able to diffuse under the cell after crossing a pore, an exact analogue to this size limit in the fluorescent assay images does not exist, although it is reasonable to assume the upper limit should be higher to account for lateral diffusion. It was frequently observed that noise clustered into objects of 8 or fewer pixels ($0.72 \mu\text{m}^2$) in area, corresponding to 0.27 μm in radius, which is smaller than the typical 1 μm radius observed in SEM. Thus, to help separate pores from noise, a minimum area of 9 pixels or more was stipulated.

The caveat of identifying pores in a green colorspace is intended to reduce variability between observers. The original staining was performed with streptavidin-488, a green stain, and the human eye is better able to detect nuanced differences in shade in the green spectrum vs other colors, meaning pore-like objects could be interpreted differently in a different colorspace. However, an arbitrary colorspace can be used, so long as any comparisons based

on visual inspection are made using an identical colorspace with identical hardware.

JRR and DRO then revisited the full set of candidate pores identified by STB, applying the above criteria. Candidate pores that were found to meet the above criteria were accepted as pores, and those which failed to meet the above criteria were rejected. Some candidates met some criteria, but failed in others (often appearing along a potential cell border). As such, "borderline" pores were candidates that could not be easily identified as transcellular pores. "Borderline" pores were thus excluded from the curated training and testing datasets, to ensure the examples used for model creation were properly labelled. "Borderline" pores were, however, included in real-world test datasets as difficult examples to more thoroughly test the recall of the models in real-world scenarios.

Cell Line	Candidate Pores Reviewed	Pass	Fail	Fail Rate
SC67	1822	594	1228	67%
SC57g	687	266	421	61%
Total	2509	860	1649	66%

Table 2.3: Numbers of pores kept and rejected following manual review process.

JRR then manually drew bounding boxes around accepted pores using the built-in "image labeller" application in MATLAB. These bounding boxes were used to extract image data at the accepted pore locations for subsequent use.

2.3.2 Manual Identification of Pores in Previously Unexamined Data

STB performed the assay on six cell lines, three normal and three glaucomatous, but only manually identified pores in SC67 and SC57g due to time constraints. JRR manually identified pores in a subset of the unprocessed image data from the remaining four cell lines. Two normal (SC65 and SC70) and two glaucomatous (SC63g and SC64g) cell lines were marked.

Marking the entire dataset was deemed impractical due to its size and the difficulties associated with the manual identification of pores. Thus, to calculate the number of images

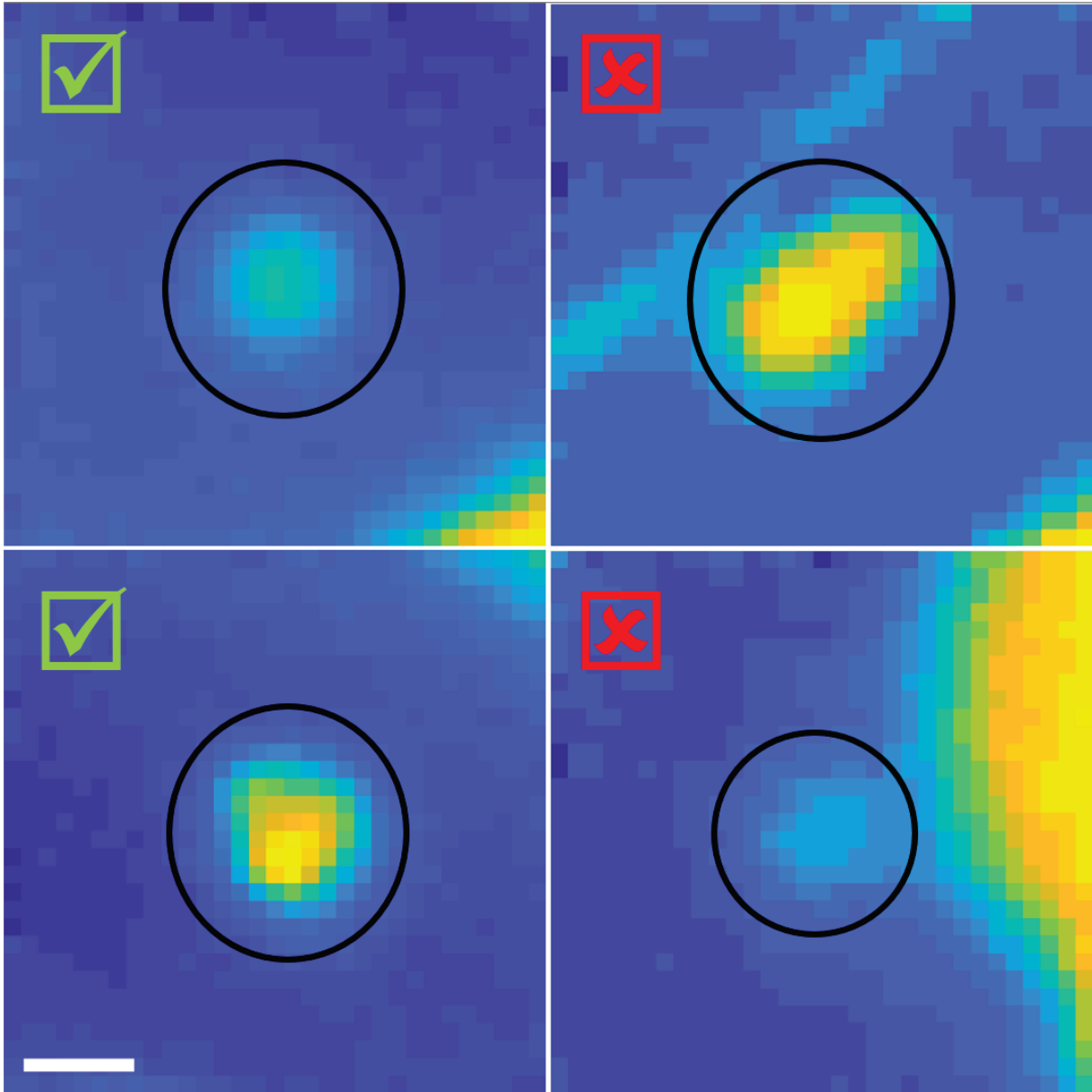


Figure 2.6: Examples of objects that would (left panels) and would not (right panels) be considered pores in the dataset based on the criteria established in section 2.3.1. The object in each frame being evaluated as pore or non-pore are indicated by black rings. Green check-marks indicate that the object in the frame meets the criteria and is considered a pore, while red crosses indicates that the object in the frame fails to meet one or more of the criteria and is thus not considered a pore. (Top Right panel) This object is considered non-pore because it is too closely associated with the border staining artefact to its upper left, as well as its high eccentricity. (Bottom Right panel) This object is considered non-pore because it is too dim relative to its immediate surrounding staining, and is too associated with the nearby border staining artefact to its right. Scale bar $\approx 2\mu m$.

necessary to detect a difference with stretch that was similar in magnitude to that reported by STB, a power calculation was used to guide subsampling of the unprocessed image data. SC pore counts are Poisson distributed in the SC67 and SC57g datasets. Based on the pore densities observed in SC67 and SC57g, which tend to be in the range of ≈ 10 pores per image, it was calculated that a sample size of 15 images per experimental condition was adequate to detect differences in SC transcellular pore density with a statistical power of 90% at $\alpha = 0.05$. The experimental conditions 0% and 20% applied mechanical stretch were selected for marking to focus on the greatest difference in pore density seen by STB in [1].

Each randomly sampled image was processed to remove obvious non-pore sections of fluorescence, such as the artefacts due to leaky junctional labelling, as described previously in section 2.2.7. New filenames were then randomly assigned to the processed images to further mask which cell line and experimental condition each sampled image belonged to. A reference key for unmasking was created to store the correspondence between original and masked filenames, to facilitate unmasking after marking was completed.

Thus, 15 images were randomly sampled for each of four cell lines and each of two experimental conditions. This resulted in a total of 120 images for marking. Marking was performed using the image labeller app in MATLAB 2021a using the criteria established in section 2.3.1. Each image was scanned manually from top left to bottom right and a bounding box was manually drawn around each identified pore present in the images. Bounding boxes were large enough to fully contain the single pore but no larger. These bounding boxes were exported when marking was complete and used to extract image data at pore locations in subsequent operations.

2.3.3 Data Partitioning

To configure the available image data into separate training and testing sets for use with machine learning algorithms, all marked images were partitioned into sets of discrete examples by applying an interest point detector. The interest point detector used was a custom processing pipeline detailed in section 2.3.4. Interest points corresponding to accepted

pores according to the established criteria were used as positive or “pore” examples for training and testing. Borderline candidate pores were left out of the training sets to avoid needless class overlap which might reduce classifier performance. The recall metric of the interest point detector was quantified as a quality control measure.

Interest points with no corresponding manually marked pore were used as negative or “non-pore” examples. This resulted in a set of several hundred thousand negative examples (see Figure 2.7 for example). To ensure training and testing was conducted using correctly labelled data, the set of negative examples was randomly sampled without replacement to give a subset of several thousand examples. This subset was manually examined to ensure all examples contained a non-pore interest point. These data were then separated into distinct training and testing sets. Positive and negative examples were split 80:20 into training and testing sets across all six cell lines.

In the case of the DS+, All6 and AugAll6 datasets, two versions of the testing dataset were created. The first, “curated”, dataset used the subset of negative examples described previously as its group of negative examples, while the second, “real-world”, dataset contained the full set of “non-pore” examples generated by the interest point detector. Pore examples remained the same between the “curated” and “full” datasets. This was done to allow for classifier performance evaluation on a vetted dataset of manageable size, while still providing the ability to test real world performance. This second larger dataset represented the entirety of the image data that would be presented to the classifier during use on unseen data, and so provided a clearer picture of real world precision.

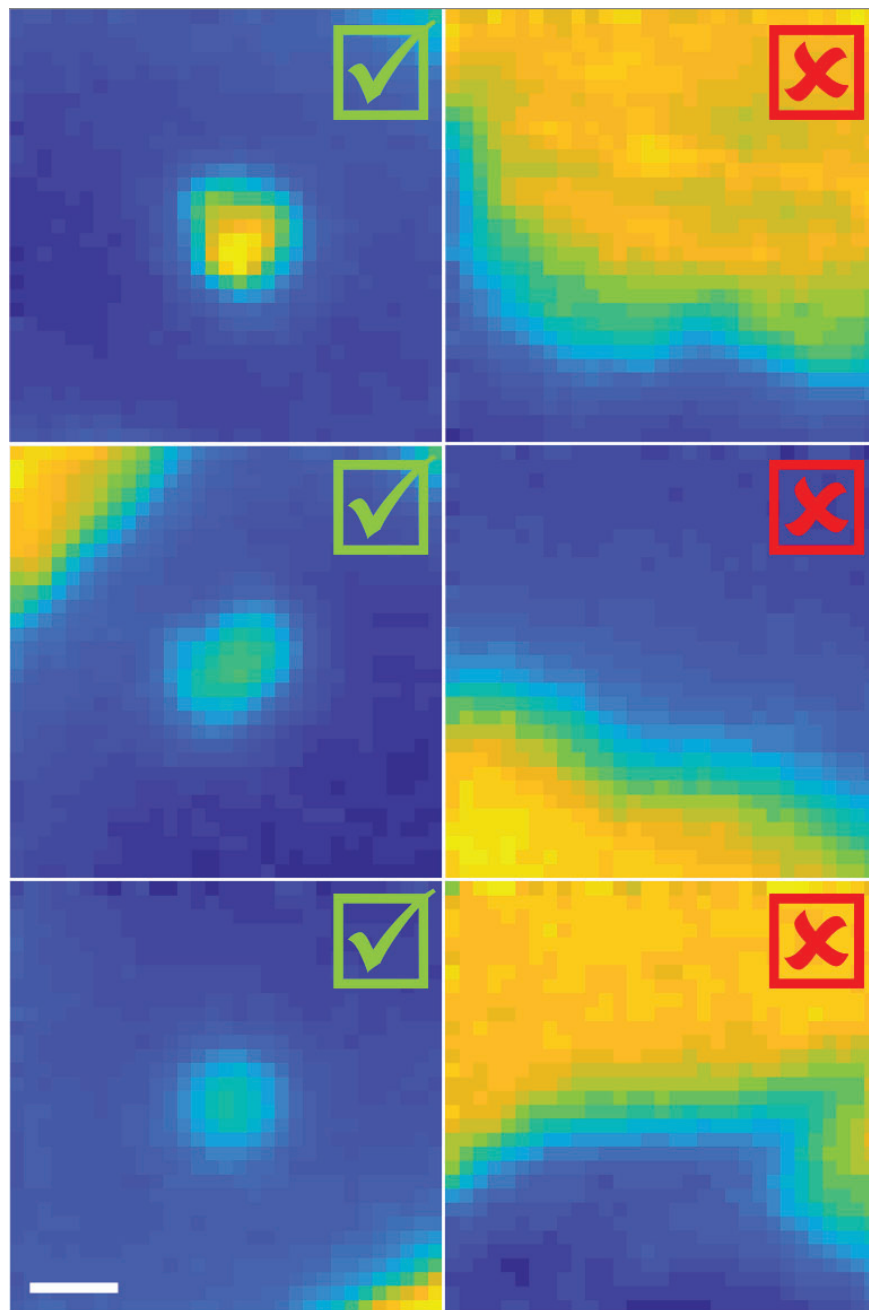


Figure 2.7: (Left panels) Examples of pores used in model training and testing. (Right panels) Examples of non-pore objects used in model training and testing. Green check-marks indicate that the object in the frame meets the criteria and is considered a pore, while red crosses indicates that the object in the frame fails to meet one or more of the criteria and is thus not considered a pore. Scale bar = $2\mu m$.

2.3.4 Interest Point Detection

A custom interest point detector was created to detect points in the raw image for further analysis (see Figure 2.8). The interest point detector first converted the RGB input image to grayscale. Large structures were then removed using a top-hat filter with a circular structuring element with a 5 pixel radius. Top hat filtering first computes the morphological opening of an image, a process that in essence removes objects smaller than the structuring element from an image while preserving the size and shape of larger objects. It then subtracts the result of opening from the original image, thereby correcting for uneven background illumination and highlighting structures of a similar scale to the structuring element used. The contrast in the resulting image was then adjusted. Very small structures were then removed by performing morphological opening, to suppress noise present in the original image and introduced by top hat filtering. The resulting image was binarized using adaptive local thresholding [44]. Segmentation was then performed on the binary image. The result of these operations was a set of locations in an image that were likely to contain a pore. The pore retention of the interest point detector, which is functionally the same as the recall metric, was quantified as a method of evaluating the viability of the interest point detector.

2.3.5 Feature Extraction

Pores typically present in the data as small blobs of fluorescence, surrounded by dark areas (ie areas with intensity close to the background level). They tend to be very circular and generally have smooth, well-contrasted edges with little textural detail (i.e. relatively uniform brightness within the blob border). Because pore shape is quite distinct and uniform, feature selection focused primarily on encoding shape based features. HOG [31] was selected for the feature extraction algorithm. Briefly, HOG works by computing the magnitudes and orientations of the gradients in an image region and binning them by angle in a lower resolution grid to produce a grid of histograms showing the distribution of edge angles of the image region (see Figure 2.9 for example). HOG encodes shape well and is commonly used in computer vision tasks for object classification tasks.

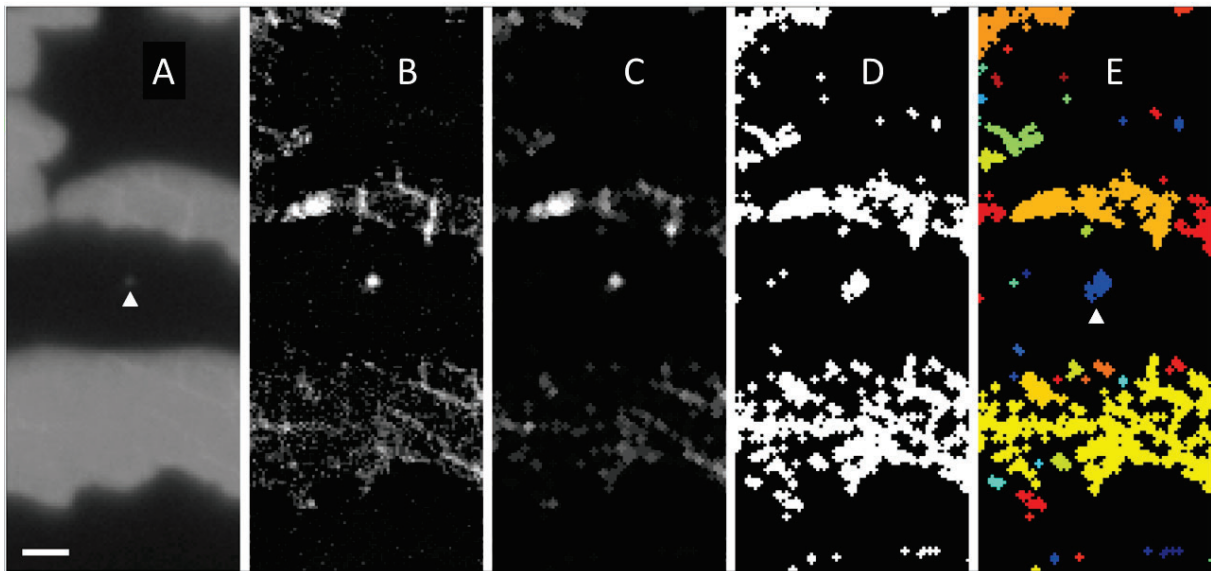


Figure 2.8: Example of interest point detection processing steps. Each panel represents the result of a processing step applied to the previous panel. A) Grayscale image region. White arrowhead indicates pore location in region. B) Results of top-hat filtering. C) Results of contrast adjustment and subsequent morphological opening. D) Results of binarization with adaptive threshold. E) Results of connected components labelling. White arrowhead indicates interest point corresponding to pore in panel A. Scale bar = $5\mu m$.

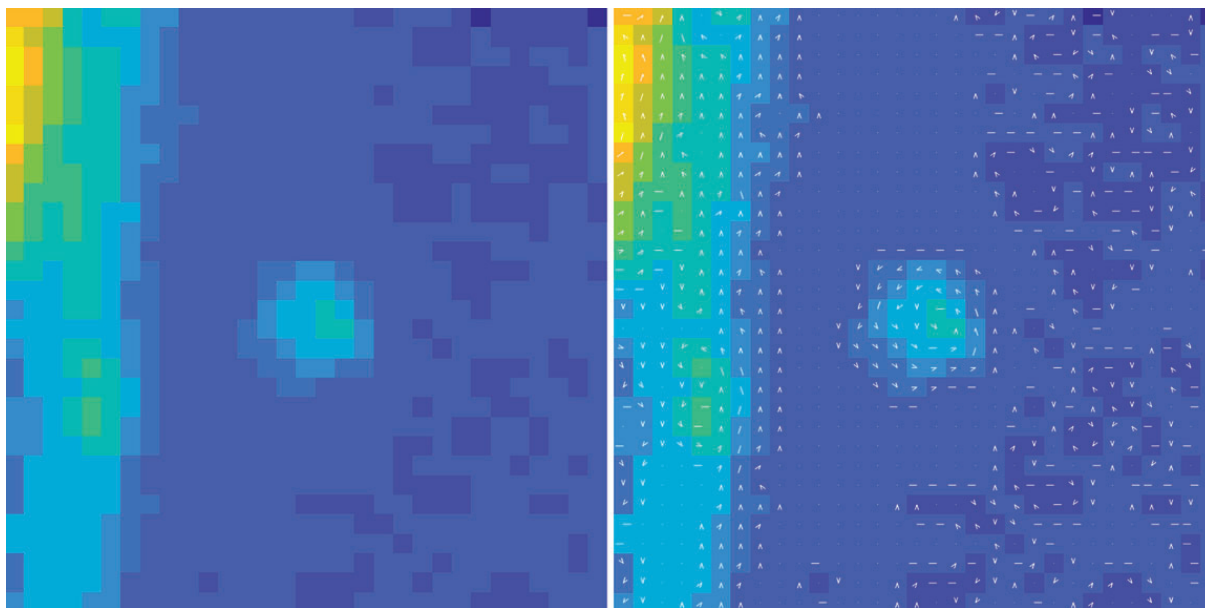


Figure 2.9: Example pore (left panel) and corresponding HOG feature data (right panel). (Right) histogram relative magnitudes and orientations visually represented with white lines.

Patches centered around each interest point's centroid were extracted from the original image, and these patches were used for feature extraction. Several patch sizes (125x125, 75x75 and 31x31) were tested. Of the options tested 31x31 displayed both the best classification performance and the shortest runtime, due to the lower number of pixels to be processed per interest point. As such, all feature extractions were performed using a 31x31 patch size.

In the AugAll6 dataset, the HOG feature data was augmented with direct measures of each interest point. Object area, solidity, eccentricity and intensity were included to better distinguish between pores and certain common artefacts (see Figure 2.10 for examples). Area allows for explicit separation of pore objects from large blob structures, such as those commonly found on cell borders, and shot noise that could be mistaken for pores based solely on shape. Solidity helps distinguish between lobed objects that may be artefacts from the staining process and actual pores. Eccentricity helps differentiate true transcellular pores from blobs that may lie along cell borders, another common type of artefact. Intensity helps to identify objects that might be otherwise porelike, but are too bright to be pores when compared to typical staining intensity within the image.

Traditional data augmentation (referred to here as "traditional" to distinguish from the

appending of shape measures used to create the AugAll6 dataset), where samples are slightly deformed to generate additional samples for training and testing, was not used. Pores in these data have few defining characteristics outside of shape, and non-pore objects in the dataset can frequently appear as pore-like but deformed from the typical pore-like shape. Because the pore class is the rare class, pores would be the subject of any traditional data augmentation applications. This would produce deformed pores that would be similar in shape to non-pore objects, effectively narrowing the feature separation between classes and thus reducing performance. For this reason, the data was not augmented with these techniques.

2.3.6 Model Selection

Five models were trained on the AugAll6 dataset. Models were selected from four different archetypes; Two boosted ensemble classifiers (RUSBoost and GentleBoost), one Gaussian SVM, one k-nearest neighbour classifier (KNN) and one decision tree. Model hyperparameters, including ensemble aggregation method and SVM kernel type, were tuned by optimizing to minimize five-fold cross validation loss. The model archetypes were selected because they are computationally inexpensive to train and run and are relatively simple to interpret. All models were trained and tested in MATLAB.

All models were trained on the AugAll6 dataset. The top three performers based on cross-validation performance were then selected for further testing on unseen data. The top three performers were applied to both the "curated" and "real-world" test sets described in section 2.3.3. The final model was selected using model performance on the "real-world" test set.

2.3.7 Performance Metric Selection

Because the data from the FM assay has a large class imbalance ($\approx 1000 : 1$), traditional performance metrics such as accuracy were not suitable for model evaluation. Instead, recall

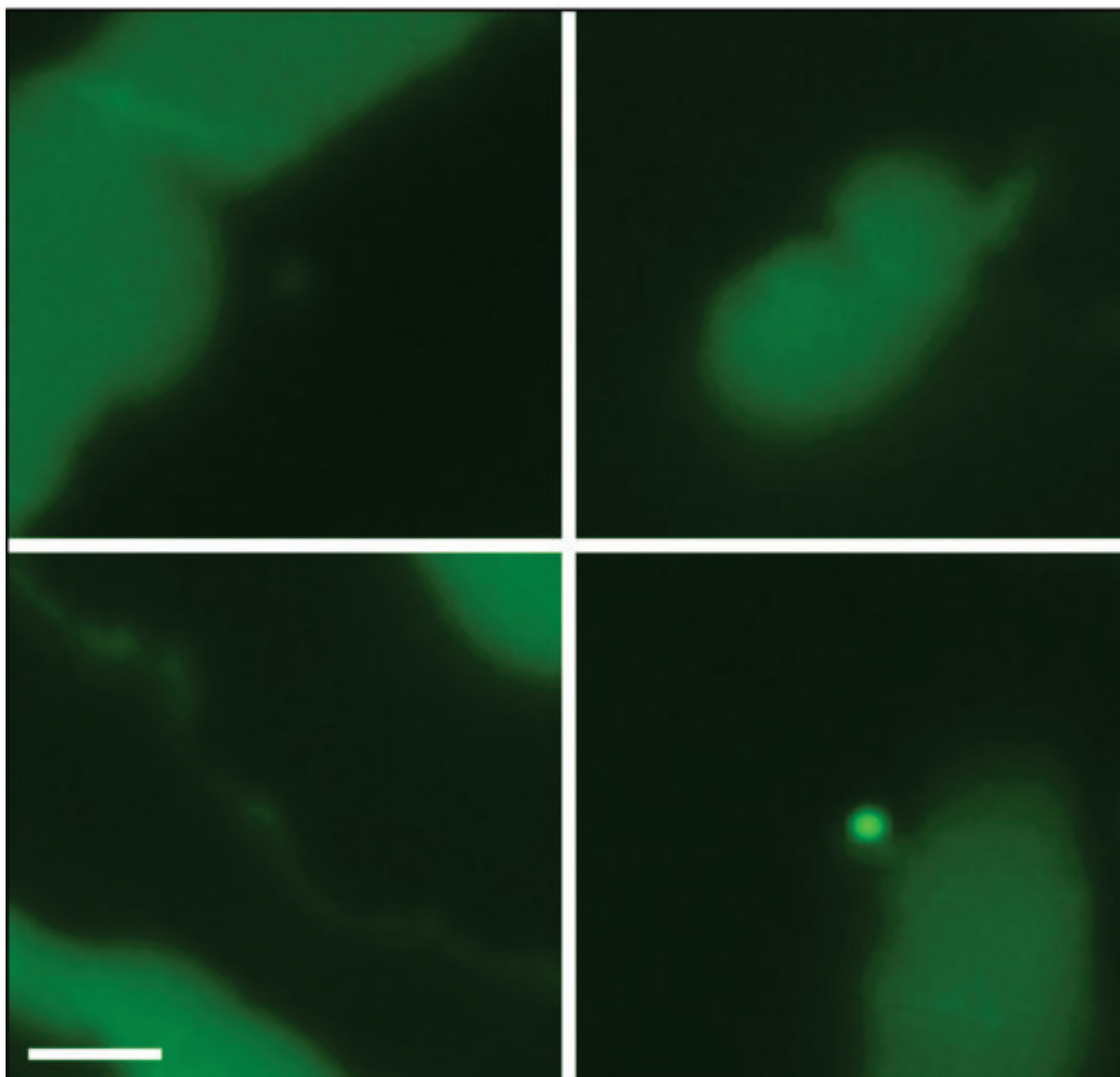


Figure 2.10: Example pore (top left panel) and non-pore objects. (Top right panel) Example of non-pore object rejected due to low solidity and large area. (Bottom left panel) Example of non-pore object rejected due to high eccentricity. (Bottom Right panel) Example of non-pore object rejected due to brightness higher than surrounding junctional staining. Scale bar = $5\mu m$

and precision on the pore class were used as indicators of model performance. F1 score was also used as an overall performance indicator.

2.3.8 Application for Manual Validation of Putative Pores

To verify the output of the pore detector algorithm, an application was written in MATLAB for the verification of pores by a human observer. The program takes as input the output of the pore detector pipeline and displays each putative pore. The user is presented with a GUI which allows the user to make a record of whether a given putative pore is a true pore or a false positive. The patch window (see Figure 2.11, section A) displays the current putative pore image patch with a high contrast contrast colormap. The full image window (see Figure 2.11, section B) displays the original image centered on and zoomed into the region containing the patch displayed in the patch window. The basic user controls section (see Figure 2.11, section C) allows the user to interact with the application to mark pores. Vote buttons allow the user to record whether the displayed putative pore is considered a true pore or a false positive. The next and previous patch buttons allow for navigation through the list of patches. An indicator light shows whether a response has been recorded for the displayed putative pore. The advanced navigation controls section (see Figure 2.11, section D) contains "jump" buttons which allow the user to quickly navigate to useful locations. Counters display current location within the list of putative pores and user progress.

Pores were randomized before display to keep the user masked. Any identifying information, such as origin cell line and image name, was also hidden to prevent user bias. The user's decisions on each pore were saved for exportation, and used to reject false positives from the dataset, leaving a final dataset consisting only of user-confirmed transcellular pores for any subsequent data analysis.

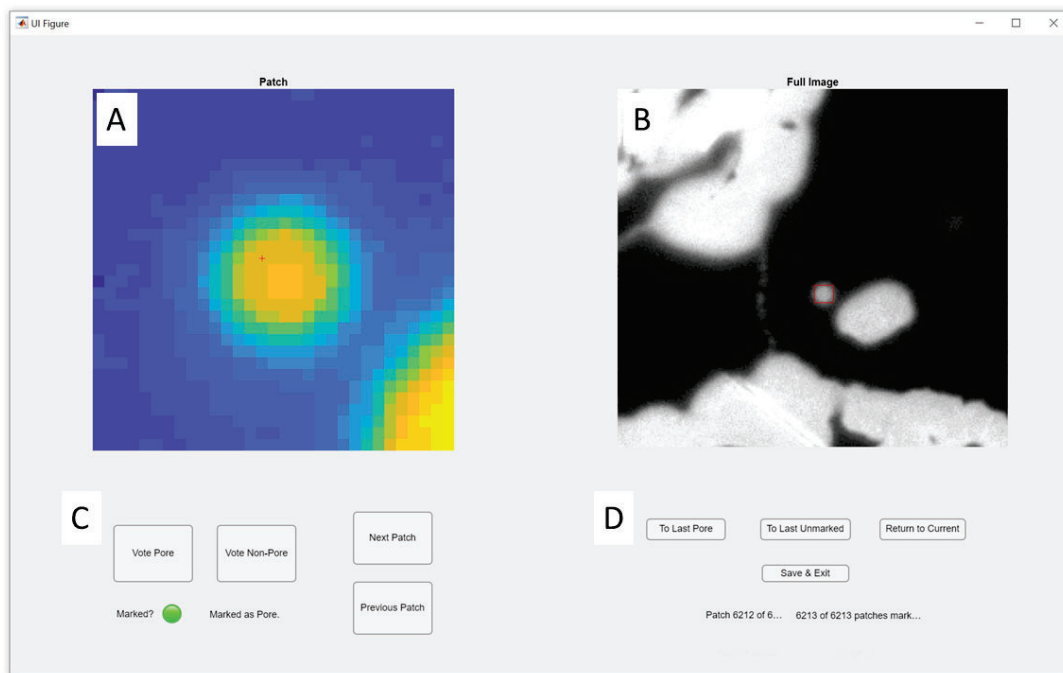


Figure 2.11: Manual validation application GUI. A) The patch window. Red + indicates the patch center. B) The full image window. Red rectangle indicates bounding box of interest point corresponding to putative pore being displayed. C) User voting and basic navigation controls. D) Advanced navigation controls.

2.3.9 Statistical Analysis

To test if pore density was different between applied mechanical strain levels or between cell lines, the E-test was applied [45]. The E-test is a modified T-test that allows for the comparison of the means of two Poisson distributions at a given significance level. Because pore density is Poisson distributed, the E-test was selected for hypothesis testing. Statistical analyses were performed in MATLAB with a significance level of 5% ($p < 0.05$);

2.4 Results

2.4.1 Interest Point Detection Recall

As a quality control test, the recall of the interest point detector was tested by applying the interest point detector to the DS+ dataset and comparing the interest points returned with the known locations of pores in the images. A pore was considered "found" by the interest point detector if an interest point was produced within the bounding box region of that pore. If no interest point was produced within a pore's bounding box, the pore was considered "missed". Thus, the recall of the interest point detector could be considered as the ratio of "found" pores to total pores in the dataset. The recall of the interest point detector was 91% in the DS+ dataset (see Table 2.3). The recall of the interest point detector was higher for SC67 at 94% versus SC57g at 88%, but met the minimum 80% recall threshold for both cell lines, set subjectively before unmasking results after discussion between JRR and DRO.

2.4.2 Model performance on DS Dataset

STB identified pores in two cell lines (one normal, SC67, and one glaucomatous, SC57g) of the six he gathered experimental data from. As a starting point, we trained and tested a RUSBoost model on the set of STB defined pores. Positive class examples consisted of the pores STB identified, while negative class examples consisted of all interest points in the

images not corresponding to a pore. The data was partitioned 80:20 by image, resulting in a training set containing 80% of the image data and a testing set containing 20% of the image data. These datasets were left unaltered, meaning that the full class imbalance present in the data was presented to the model during training and testing. The resulting model, named "Digital Sietse" (DS), which contained the full set of STB defined pores, showed poor testing performance with an F1 score of 0.0072, mainly due to its extremely low precision (see Table 2.4).

Model Name	DS RUSBoost
F1 Score	0.0072
Recall	72%
Precision	0.4%
FPR	0.98

Table 2.4: Performance of the RUSBoost model trained on the DS dataset, consisting of all pores identified by STB in data from cell lines SC67 and SC57g.

2.4.3 Model performance on DS+ Dataset

The DS RUSBoost model's performance was unsatisfactory, so we sought methods of improving performance. To this end, we conducted a formal review of the pores identified by STB, as detailed in section 2.3.1, keeping only the pores that passed this review. From the pore data that passed review, we created new training and testing datasets, as detailed in section 2.3.3. These datasets were curated, meaning they contained the verified pore examples and a subset of the available non-pore examples. These sampled non-pore examples were also vetted manually, meaning the number of total examples was far fewer than in a "real-world" scenario, since it would be infeasible to review the hundreds of thousands of negative examples in a full dataset. It also meant that the class imbalance presented during training and testing was far less than in the full "real-world" dataset.

To address this, we took the pore examples from the "curated" test dataset and made a separate and additional "real-world" test dataset that contained all the negative examples in the images those test pores were derived from. This allowed us to test model performance on

a dataset with no labelling errors, while also providing insight into how the model would perform on "real-world" data due to the class imbalance.

The model resulting, named "Digital Sietse +" (DS+) to reflect that it was created from a refined version of the original data, showed drastic performance improvements on the test set (see Table 2.5), with an F1 score of 0.88. However, "real-world" performance was found to be severely lacking, with an F1 score of 0.18 and very poor recall (see Table 2.6).

Model Name	DS+ RUSBoost
F1 Score	0.88
Recall	79%
Precision	99%
FPR	0.0005

Table 2.5: Test performance of the RUSBoost model trained on the DS+ dataset. Results are derived from the curated DS+ testing set.

Model Name	DS+ RUSBoost
F1 Score	0.18
Recall	41%
Precision	11%
FPR	0.007

Table 2.6: "Real-world" performance of the RUSBoost model trained on the DS+ dataset. Results are derived from the uncurated, "real-world" DS+ testing set.

2.4.4 Model performance on the All6 Dataset

While the DS+ RUSBoost model was an improvement over the DS RUSBoost model, its "real-world" performance was insufficient. We attempted to improve performance further by including more data in training and testing. Because the DS+ dataset already used all the available pore data identified by Sietse, we had to manually identify pores in the other four cell lines STB collected experimental data from. We manually identified pores in two normal (SC70, SC65), and two glaucomatous (SC63g, SC64g) cell lines, as detailed in section 2.3.2. These pores were added to the DS+ dataset, along with a subset of the non-pore examples from the unused images, to create new curated training and testing sets, as well as a new "real-world" test set, as detailed in section 2.3.3. These datasets were named "All6" to reflect that it was created using data from all six cell lines.

The All6 RUSBoost model showed further improvement over the DS+ RUSBoost model on the curated test set (see Table 2.7). "Real-world" performance also saw a substantial boost over the DS+ RUSBoost model, but recall was still too low for a useable implementation (see Table 2.8).

Model Name	All6 RUSBoost
F1 Score	0.93
Recall	92%
Precision	94%
FPR	0.005

Table 2.7: Test performance of the RUSBoost model trained on the All6 dataset. Results are derived from the curated All6 testing set.

Model Name	All6 RUSBoost
F1 Score	0.11
Recall	65%
Precision	6%
FPR	0.01

Table 2.8: "Real-world" performance of the RUSBoost model trained on the All6 dataset. Results are derived from the uncurated, "real-world" All6 testing set.

2.4.5 Model performance on the AugAll6 Dataset

The All6 RUSBoost model still required further improvements in real-world performance, so we attempted to boost performance further. To do this, we augmented the All6 dataset with explicit shape metrics of each interest point, to attempt to explicitly encode the criteria from section 2.3.1 into the feature data, since these metrics were sufficient to distinguish between pore and non-pore objects manually. The resulting dataset was named "AugAll6" to reflect that it was simply the All6 dataset augmented with additional information.

The AugAll6 RUSBoost model again showed excellent performance on the curated test dataset, with the best F1 score yet of 0.94 (see Table 2.9). Importantly, recall and precision also increased in real-world testing (see Table 2.10).

Model Name	AugAll6 RUSBoost
F1 Score	0.94
Recall	92%
Precision	97%
FPR	0.003

Table 2.9: Test performance of the RUSBoost model trained on the AugAll6 dataset. Results are derived from the curated AugAll6 testing set.

Model Name	AugAll6 RUSBoost
F1 Score	0.15
Recall	89%
Precision	9%
FPR	0.008

Table 2.10: "Real-world" performance of the RUSBoost model trained on the AugAll6 dataset. Results are derived from the uncurated, "real-world" AugAll6 testing set.

2.4.6 Identification of the Optimal Model Architecture using the AugAll6 Dataset

Performance on AugAll6 Curated Test Dataset

Once the AugAll6 RUSBoost model was found to have sufficient performance as a pore detection tool, we next sought to verify whether other classification algorithms might give better performance, despite RUSBoost's explicit modifications to handle imbalanced data. To this end we trained and tested other model architectures on the AugAll6 dataset as detailed in section 2.3.6 and compared them to the AugAll6 RUSBoost model.

All five models displayed excellent cross-validation performance, with F1 scores above 0.90 for all models trained (see Table 2.11). The three best performers were GentleBoost, Gaussian SVM and RUSBoost, with F1 scores well above 0.95. These three models also all performed well on the 'curated' test set, where performance was very competitive across the board (see Table 2.12).

Model	Recall	Precision	F1 Score	FPR
GentleBoost	97.8%	98.8%	0.983	0.004
Gaussian SVM	98.0%	98.2%	0.981	0.01
RUSBoost	96.4%	97.9%	0.971	0.01
KNN	93.6%	96.7%	0.951	0.01
Decision Tree	92.5%	94.0%	0.932	0.02

Table 2.11: Cross-Validation performance of various models on the AugAll6 curated training dataset. All performance metrics are reported on the positive (pore) data class.

Model	Recall	Precision	F1 Score	FPR
GentleBoost	93.9%	98.0%	0.9569	0.0021
Gaussian SVM	93.4%	96.1%	0.9476	0.0034
RUSBoost	91.6%	97.0%	0.9420	0.0025

Table 2.12: Performance of models on the AugAll6 curated testing dataset. Models applied are the top performers according to cross-validation results. All performance metrics are reported on the positive (pore) data class.

Cell Line	FN	TN	FP	TP	Recall	Precision	F1 Score
SC70	4	54,570	451	27	87.1%	5.65%	0.1061
SC65	2	56,803	370	23	92.0%	5.85%	0.1100
SC64g	1	95,638	203	9	90.0%	4.25%	0.0811
SC63g	2	81,287	99	19	90.5%	16.1%	0.2734
SC57g	14	45,577	1,578	123	89.8%	7.23%	0.1338
SC67	2	111,315	1,117	105	98.13%	8.59%	0.1580
Total	25	445,190	3,818	306	-	-	-
Average	-	-	-	-	91.3%	7.95%	0.1437
Std Dev	-	-	-	-	3.40%	3.89%	0.0627

Table 2.13: Gaussian SVM performance on the "real-world" AugAll6 testing dataset. Performance is broken down by cell line. Raw counts used to compute recall, precision and F1 Score are shown in columns 2-5. Average performance metrics and standard deviation across cell lines are shown in rows 9 and 10 respectively.

Cell Line	FN	TN	FP	TP	Recall	Precision	F1 Score
SC70	4	54,481	540	28	90.3%	4.93%	0.0935
SC65	2	56,779	394	23	92.0%	5.52%	0.1041
SC64g	1	95,709	132	9	90.0%	6.38%	0.1192
SC63g	3	81,303	83	18	85.7%	17.82%	0.2951
SC57g	17	45,574	1,712	121	87.7%	6.60%	0.1228
SC67	4	111,313	1,119	104	96.3%	8.50%	0.1563
Total	30	445,159	3,980	303	-	-	-
Average	-	-	-	-	90.3%	8.29%	0.1485
Std Dev	-	-	-	-	3.34%	4.40%	0.0684

Table 2.14: GentleBoost performance on the "real-world" AugAll6 testing dataset. Performance is broken down by cell line. Raw counts used to compute recall, precision and F1 Score are shown in columns 2-5. Average performance metrics and standard deviation across cell lines are shown in rows 9 and 10 respectively.

Cell Line	FN	TN	FP	TP	Recall	Precision	F1 Score
SC70	4	54,492	529	27	87.1%	4.86%	0.0920
SC65	3	56,796	377	22	88.0%	5.51%	0.1038
SC64g	1	95,734	107	9	90.0%	7.76%	0.1429
SC63g	4	81,308	78	17	81.0%	17.89%	0.2931
SC57g	16	45,575	1,627	112	87.5%	6.44%	0.1200
SC67	2	111,315	1,087	103	98.1%	8.66%	0.1591
Total	30	445,220	3,805	290	-	-	-
Average	-	-	-	-	88.6%	8.52%	0.1518
Std Dev	-	-	-	-	5.07%	4.38%	0.0671

Table 2.15: RUSBoost performance on the "real-world" AugAll6 testing dataset. Performance is broken down by cell line. Raw counts used to compute recall, precision and F1 Score are shown in columns 2-5. Average performance metrics and standard deviation across cell lines is shown are rows 9 and 10 respectively.

Performance on AugAll6 Real-World Test Dataset

In "real-world" testing, performance, and particularly precision, drops starkly across the board (see Tables 2.13, 2.14 and 2.15). Notably, the precision of RUSBoost goes from the lowest among the three top performers to the highest on real-world data, which is likely due to its explicit modifications to accommodate class imbalance.

The precision of even the best performing model is too low for a completely automated deployment, since an 8% precision would result in roughly 11 false positives reported for every pore correctly identified. However, the model itself is capable of classifying pores with high precision, as demonstrated by performance on the curated testing dataset. Rather, it is the large class imbalance of real-world datasets that causes the large number of false positives, which lowers precision. To deal with this, the results from the pore detector should be manually validated by a human observer, as detailed in section 2.3.8. This would mean the pore detection algorithm would be used as an advanced pre-screening tool. The user would then be shown the pores detected by the algorithm and would vet each putative pore using the criteria established in section 2.3.1.

Average recall can be considered roughly equal across all three models (see Tables 2.13, 2.14 and 2.15), since the number of positive examples is small. The small advantage in precision gained by RUSBoost is more significant, due to the large numbers of false positives. The lower the precision, the more time must be spent manually validating the pore detector's output. Because recall between models can be considered equal, we thus selected the model with the best precision as the best performing model, since the opportunity cost of losing a small number of pores with a lower recall model is outweighed by the time-savings of having fewer examples to manually review. This makes the RUSBoost model trained on the AugAll6 dataset the best performing model.

2.4.7 Manual Validation Performance

Because the pore identification pipeline is intended for deployment with a manual validation end step, we sought to quantify the accuracy of this step of processing. To this end, we provided the manual validation application described in section 2.3.8 to a non-expert subject along with a set of 400 objects comprising an equal number of pore and non-pore objects. The criteria established in section 2.3.1 were also provided to the subject, along with a brief ten minute training session demonstrating the proper application of said criteria. All objects provided for performance testing were labelled according to the aforementioned criteria.

After training was complete, the subject was allowed to perform manual validation on their own time, and the results were returned for analysis after completion. On the dataset provided, the subject scored well, with an accuracy of 75%. However, while no false positive errors were made by the subject, 50% of pores in the dataset were rejected, impacting recall.

2.5 Discussion

SC inner wall pores are thought to be a key site of AH outflow resistance, and thereby IOP, regulation. SC inner wall cells obtained from glaucomatous human donor eyes have been shown to have a reduced capacity to form pores, and this impairment is maintained in culture. This means that the study of SC inner wall pores is of great interest and importance to glaucoma research. It is therefore of great importance to have a method of studying pores at large scales quickly and objectively.

The fluorescent pore assay provides a method of studying pores at large scale, but only if the data obtained from the experiments can be processed in a timely and objective manner; in other words, the usefulness of the experimental method is bottlenecked by the method of data analysis. Manual processing takes days to weeks to analyze single cell lines. This does not scale when considering the desire to test possible therapeutics on cultured SC cells to see their effect on pore formation, rapidly increasing the number of datasets that must be analyzed.

The subjective nature of identifying pores, due to the possible variance in imaging conditions and observer interpretation of pore definitions, also hinders the usefulness of any analysis done on the data, as it becomes difficult to compare the analysis of different researchers.

A method of automatically processing the data obtained from the fluorescent pore assay is thus very desirable and arguably a requirement to begin large scale studies into the glaucomatous impairment of SC pore formation and potential methods for reversing said impairment. The system detailed here is a leap towards that goal, screening in minutes the number of images that would take days or weeks to process manually. The poor performance of the prototype models presented above, trained on the DS, DS+ and All6 datasets, indicate the importance of having well labelled data with vetted high quality examples, as well as the importance of maximizing the amount of data used for model training and the importance of feature selection. The models trained on the AugAll6 dataset presented have high discriminative capacity on balanced datasets, indicating that the problem of pore classification can be well handled by machine learning systems. In a vacuum based on the "curated" test set, it would appear that the models presented here are all suitable for deployment, but unfortunately even the best models presented have low "real-world" precision due to the high class imbalance inherent to the dataset and to SC pore detection as a computer vision task. For a fully automated pore detection paradigm, the class imbalance of the data is a major hurdle that must be overcome.

Despite this, we believe that the AugAll6 RUSBoost model, which has the best "real-world" precision and best F1 score on the "real-world" pore dataset of the models tested and is explicitly designed to deal with imbalanced class data, is deployable, but that it must be accompanied by some form of manual quality control process due to its low precision. We propose that the pore detection pipeline be applied to data as described above, using the AugAll6 RUSBoost model, and that the output of the pore detection pipeline be treated as putative pore locations. These putative pores should then have their identifying information (such as parent image, cell line, experimental condition) masked. This set of masked putative pores should then be randomized and presented to a manual observer for final classification, using the criteria established above. As the results from manual validation testing show,

special attention should be given to the training of users of the manual validation application, to ensure the pore identification criteria are interpreted similarly between observers.

While it may be possible in the future to solve the issue of the class imbalance in such a way that makes the manual validation procedure described above unnecessary, in its current form the pore identification system presented above provides a way of decreasing the time of data analysis from weeks to hours while increasing the objectivity of the data analysis process, enabling researchers to begin investigating glaucomatous SC pore formation impairment at greater scale by cutting down on the image area to be manually inspected through the presentation of putative pores, as opposed to brute-force whole image searching.

Another advantage of utilizing the pore detector pipeline is that pores can also be analyzed in terms of their dimensions, geometry and other visual properties with minimal additional processing cost, which is not possible at scale with manual pore identification. The measurements taken can be customized to the task and require negligible additional processing time to compute, further enhancing workflow over manual processing. This allows for easy comparison of metrics such as pore area between different experimental conditions, which may provide insight into why outflow resistance is increased in glaucoma.

A key area for the improvement of pore detection will likely be the culture of SC cells and the fluorescent assay themselves. SC cells do not form tight junctions in culture, which leads to labelling artefacts in the image data that make pore identification difficult, due to their high intensity, size and frequency relative to pores. These artefacts make paracellular pore identification impossible and transcellular pore identification much more difficult. Improving the junctions would greatly reduce the complexity of the images and make pore identification much easier. Any method that reduces labelling artefacts, whether by improving tight junction formation in SC culture or by modifying the stain to label leaky junctions less, will reduce the number of interest points to be investigated, which effectively reduces the class imbalance seen by the classifier, as well as further improving processing time by reducing the number of locations where feature data must be extracted.

Another option for improving the performance of the pore detector pipeline may be to switch

to using subconfluent culture of SC cells, rather than the confluent culture technique currently used. If SC pore formation is maintained not just in culture, but in subconfluent culture, the issue of border artefacts can be turned from a hinderance into a strength, by providing clear borders for each cell. This would allow for studies on the locations of pores within cells, providing greater insight into SC pore formation, and possibly the underlying subcellular structures that govern the transcellular pore formation process, via co-localization studies. Subconfluent culture may also provide other benefits, such as the ability to control cell area and thickness through the use of micropatterned substrate coating. It should be noted, however, that any subconfluent study will not be able to investigate paracellular pore formation, and that a method of studying paracellular pore formation at scale does not currently exist. Subconfluently cultured SC cells also fail to replicate the high aspect ratio observed in SC cells in situ and in confluent culture, and the effect this has on pore formation is currently unknown.

Chapter 3

Trends in Schlemm's Canal Inner Wall Transcellular Pore Density, Area and Shape

3.1 Introduction

Glaucoma is the leading cause of blindness worldwide [2], and is associated with elevated IOP, most commonly caused by an increase in outflow resistance. The majority of conventional outflow resistance in primates is generated in the immediate vicinity of the inner wall of SC [5]. As AH flows out of the eye, the SC inner wall is perfused by AH in the basal-to-apical direction, causing the endothelial cells to be pushed off their basal lamina and into the SC lumen. This pressure drop deforms the cells, and SC inner wall cells form pores in response to cellular deformations [8], a behavior that is retained in culture. These pores are the route by which AH drains from the COP into the SC lumen [9], and as such are likely highly important in outflow resistance regulation. SC pore density is reduced in glaucoma [10],[11], which may help explain the increase in outflow resistance that causes the elevated IOP associated with the disease, and SC pores are thus a key area of focus for glaucoma research.

In an effort to produce a high-throughput method of studying SC inner wall pore density, a fluorescent assay to label transcellular pores in cultured inner wall SC cells was created [1]. This assay produces large amounts of image data, which must then be processed to extract useful information. This analysis would previously be done manually, but manual analysis presents several challenges, such as impractical time costs and observer bias. To combat these issues, we created a purpose built machine learning based pore detection software pipeline to facilitate data analysis, detailed in Chapter 2. The pore detection pipeline examines the images and produces a list of possible pore locations, based on prior knowledge of typical pore appearance in the data. This greatly reduces the time needed to examine a given image for pores, allowing for more images, and so a greater culture area, to be examined.

The possible pore locations reported by the pore detection pipeline are then reviewed manually using a manual validation application (see section 2.3.8), which presents each location to the observer in a masked and randomized manner and records the observer's final decision on whether the location contains a pore. The enforced anonymized environment provided by the manual validation application addresses variability and combats observer bias by providing a standardized method of examining pores. The established set of criteria (detailed in section 2.3.1) also helps to standardize pore identification. Lastly, the pore detection pipeline is also capable of extracting pore area and shape measures, allowing for deeper investigation into SC pore attributes, such as size, shape and density, than was previously feasible, and can be easily modified to report virtually any visually based measurement of pores.

Here we apply the pore detection pipeline and manual validation process to a random subsampled set of image data acquired from previous experiments (see section 2.2 for details on experimental procedure). We examine six "cell lines" (three normal (SC65, SC67 and SC70) and three glaucomatous (SC57g, SC63g and SC64g)), identifying pores in an average culture area of 3.63 mm^2 per cell line. Using the pores identified, we then investigate whether SC inner wall transcellular pore density varies with applied mechanical strain across cell lines. We also leverage the measurements taken by the pore detection pipeline to investigate whether SC inner wall transcellular pores exhibit different morphological characteristics, such

as area and shape metrics, between cell lines or disease state.

3.2 Methods

3.2.1 Data Preparation

The experiments conducted by STB (detailed in section 2.2) produced 1,359 images across three cell lines, each with three mechanical strain levels (0%, 10% and 20%). Note that, while the cell cultures for this study would typically be referred to as cell strains due to their nature as primary cell cultures, we use the term "cell line" to avoid confusion between mechanical "strain" and cell "strain". Because the pore detector has low precision in real-world datasets, the total number of images from all cell lines was deemed too large for user validation. To mitigate this, we opted to subsample the image data and analyze this subset.

The number of images to be sampled was determined by applying the pore detector to all the available image data and estimating the number of putative pores expected for a given number of images per stretch condition per cell line. To investigate whether the data showed an increase in pore density with stretch, we sampled data from the three normal cell lines (SC65, SC67 and SC70) at 0% and 20% mechanical strain. To reduce the burden of manual user validation, the 10% strain data was not examined in this study.

To investigate whether pore density increased less with stretch in glaucomatous cells than in normal cells, we chose to sample from the three glaucomatous cell lines (SC57g, SC63g and SC64g) at 20% strain for comparison with the normal cell lines at 20% strain. Based on the experimental conditions selected for examination and the expected number of putative pore locations per image, a sample size of 30 images per experimental condition was selected¹, corresponding to an estimated 6700 putative pores to review, for a total of 270 images.

¹The sample size of 30 images per experimental condition was selected by applying the pore detector to the entire dataset of 1,359 images and noting how many putative pores were returned per image. This number was used to select a number of images that would produce a manageable number of putative pores to review. This number is well above the minimum determined via the power calculation detailed in section 2.3.2 and so was considered suitable.

All images were examined prior to sampling for quality. Images with severe artefacting, such as out-of-focus, overexposed or damaged regions, were not considered for sampling, as the artefacting rendered large sections of the images impossible to analyze (see Figure 3.1. For some experimental conditions this exclusion of artefacted images meant that fewer than the targeted 30 images were available for sampling. In this case, all images for that experimental condition remaining after excluding artefacted images were included for analysis. This resulted in a final total of 241 images being sampled for analysis, with an average examined area per experimental condition of 3.63 mm^2 and a minimum area examined per experimental condition of 1.36 mm^2 .

Cell Line	Strain (%)	# Images	Image Area (mm^2)
SC67	0	25	3.39
	20	30	4.07
SC65	0	10	1.36
	20	30	4.07
SC70	0	30	4.07
	20	30	4.07
SC57g	20	26	3.53
SC63g	20	30	4.07
SC64g	20	30	4.07

Table 3.1: Breakdown of number of images and area examined per experimental condition and cell line. Note that strain here refers to mechanical strain applied to cells during experiments.

These images were masked for the study by using a MATLAB script to copy the images to a separate folder and rename them so as to obscure from the observer which experimental condition and cell line they originally came from. A key was generated to allow the results to be unmasked after manual validation was completed.

3.2.2 Pore Identification

The masked images were analyzed using the pore detector, described in section 2.3 (see Figure 3.2). Briefly, the pore detector is a pipeline of several processing algorithms, designed to detect pores in SC cell image data obtained using the pore staining assay developed by STB in [1]. Input images are subjected to a series of processing steps designed to detect interest points in the images. These interest points are then passed to a feature extractor,



Figure 3.1: Examples of images that were rejected due to severe artefacting during the examination process. (Top panel) Example of overexposure. (Bottom panel) Example of damaged regions. Scale bar approximately = $30\mu m$

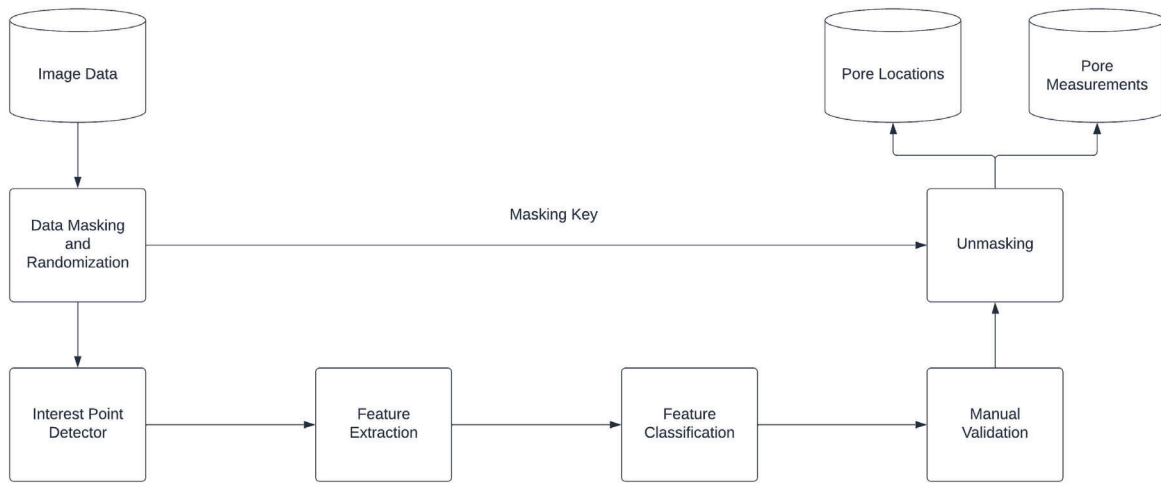


Figure 3.2: Flowchart depicting the processing steps of the pore detection pipeline.

where HOG features are extracted from the local area around each interest point and augmented with explicit measures of the interest point. These feature data are then passed to a RUSBoost classifier, trained on the "AugAll6" dataset (created in section 2.3), for classification. The result is a series of putative pore locations within the images, and a set of measures extracted from each point.

These putative pore locations are then loaded in a manual validation application, described in section 2.3.8 (see Figure 2.11). The manual validation application presents the putative pore locations in a random order and masks identifying information, such as the origin cell line, experimental conditions and disease state associated with each pore, before displaying the putative pores to the user. The user enters the final decision on whether each putative pore is a true transcellular pore, based on the criteria established in section 2.3.1, and the application records this information. When manual validation is complete, the record of the user's decisions are exported and combined with the pore detector's output to produce an unmasked final list of pores present in the data and any associated measures of those pores.

3.2.3 Pore Measurements

The pore detector was augmented to report several measurements of each pore identified, to allow analysis of SC transcellular pore morphology as well as density. Pore density, area, eccentricity and solidity were investigated. The obtained measurements were validated by randomly selecting a small sample of pores and performing manual measurements in ImageJ. Comparison between manually and automatically acquired measures showed no discernable difference.

Area is here defined as the number of pixels belonging to the identified object, after connected components labelling. This is converted to the units of mm^2 by multiplying the number of pixels with the area per pixel value ($0.0986\mu\text{m}^2/\text{px}$ for all images²) in the metadata of the image the pore is derived from.

The eccentricity of a region is defined as the ratio of the distance between the foci of an equivalent ellipse³ of the region and the region's major axis length (see Figure 3.3, top panels for examples). This produces a value between 0 and 1, where a region with an eccentricity of 0 is a circle and a region with an eccentricity of 1 is a line segment. This effectively gives a measure of pore elongation versus roundness that can be applied to very small areas without becoming unbounded, which is not the case for the more commonly used circularity. Because pores can have very small areas (as low as 8 pixels in extreme cases), this quality makes eccentricity preferable to circularity for measuring pore roundness.

Solidity is defined as the ratio of pixels in the convex hull of an object that also exist within that object's perimeter (see Figure 3.3, bottom panels for examples). This value can be interpreted as the concavity of the object, and has a maximum of 1, where an object with a solidity of 1 is a closed circle.

²This value is particular to the images examined in this study. When applying the pore detector to other datasets, the area per pixel must be known in order to produce area measurements in physical units.

³To create the equivalent ellipse of a binary region, the covariance matrix of the region is calculated by computing the second central moments of the object. The eigenvectors and eigenvalues of this covariance matrix are the directions and magnitudes, respectively, of the major and minor axes of the equivalent ellipse. The magnitudes of the major and minor axes are then used to calculate eccentricity according to $\sqrt{1 - (\frac{b}{a})^2}$, where a and b are major and minor axis length, respectively.

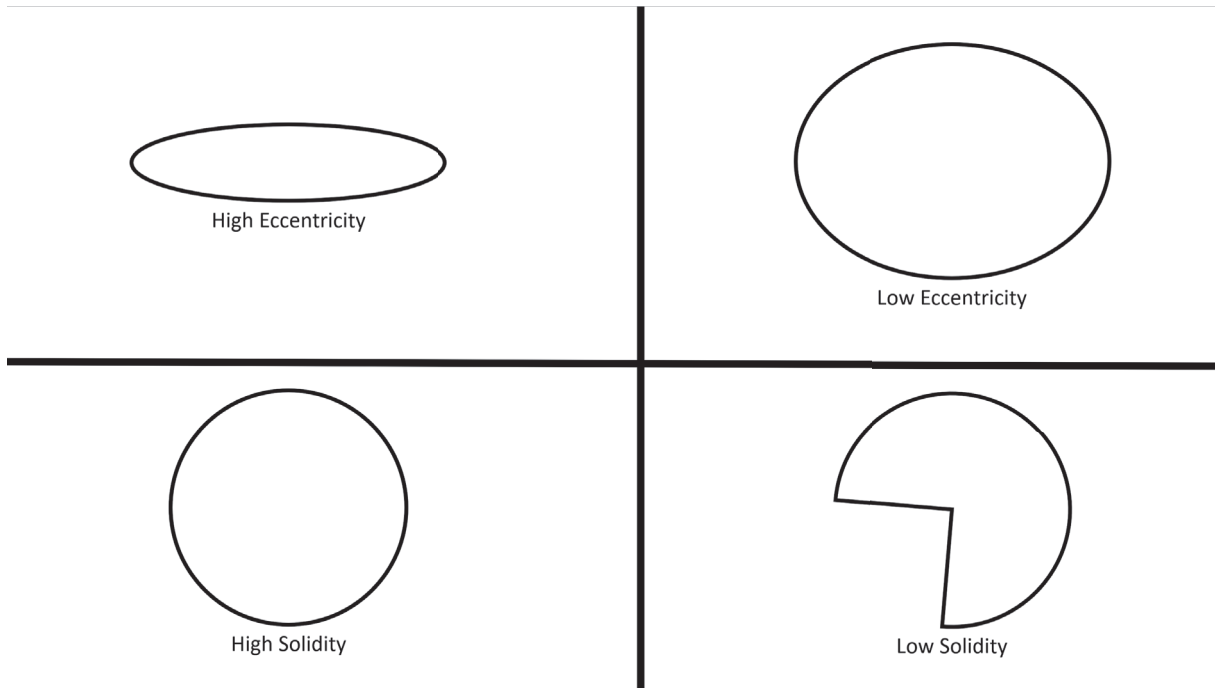


Figure 3.3: Diagrammatic examples of high and low eccentricity and solidity objects.

3.2.4 Outflow Resistance Estimation

To investigate the effect of pore area and density on outflow resistance, we utilized the measured pore area and density values to calculate the outflow resistance attributable to the inner wall, assuming that flow only passes through transcellular pores having the same density and distribution of diameters as measured in the images. The following calculations assumed that the observed pore density was uniform across the entire inner wall area of 11mm^2 [46].

The measured value of pore area A_p was converted to an effective pore diameter d by approximating pores as circles and taking $d = \sqrt{\frac{4A_p}{\pi}}$. Because pore area is log-normally distributed and pore diameter is derived from pore area, we assumed that pore diameter would also be log-normally distributed. Thus, to report the average pore area and diameter, we determined the means of the log (base 10) transformed distributions, and then calculated the exponent of this mean (using base 10) to reverse the transformation and determine the corresponding values of the mean area and diameter in the linear domain.

Outflow resistance attributable to inner wall pores can be calculated by assuming viscous flow

through a circular orifice having resistance $R = \frac{24\mu}{d^3}$, where μ is aqueous humor viscosity and d is pore diameter. For a number of orifices in parallel, the total resistance can then be calculated as $R = \frac{24\mu}{d^3 n A_{IW}}$, where A_{IW} is the area of the inner wall and n is pore density [6]. This model assumes a uniform pore density and third moment of diameter across the entirety of the inner wall.

Because we captured pore diameter for populations of pores, we adapted the above expression to take this into account. To this end, we calculated the third moment of the pore diameter distribution for a given experimental condition (reported in Table 3.3 as “ nd^3 ”) as $\frac{1}{A_E} \sum_{i=0}^N d_i^3$, where d_i is the effective diameter of the i^{th} pore identified for that experimental condition, and A_E is the total image area examined for that experimental condition. This approach better modeled the filtration area by considering the population of pores to be a set of parallel resistors to flow over the observed area.

For completeness, we also report the first and second moments of the pore diameter distributions, following from [9]. These moments were calculated in a similar fashion to the third moment described above, and correspond to the effect of pore density and diameter on outflow resistance according to the funneling and porosity models respectively.

3.2.5 Statistical Tests

One-sample Kolmogorov-Smirnov tests were used to test for normality. Pore counts were tested for Poisson distribution via chi squared goodness of fit. Because pore counts are Poisson distributed, the E-test [45] was used to compare pore count distributions between experimental conditions. Nested analysis of variance (ANOVA) was used to test for differences between groups in normally distributed data. Data was nested in the heirarchy of stretch level within cell line within disease state. Where appropriate, a Bonferroni correction was applied to adjust p values to reduce the chance of false positive errors due to the application of multiple pair-wise statistical tests to the same dataset. All statistical analyses were performed in MATLAB.

3.3 Results

3.3.1 Pore Density Trends

Cell Line	Strain (%)	Pore Count	# Images	Image Area (mm ²)	Pore Density (mm ⁻²)
SC65	0	12	10	1.36	8.84
	10	40	26	3.53	11.34
	20	84	30	4.07	20.63
SC67	0	141	25	3.39	41.56
	10	239	30	4.07	58.71
	20	272	30	4.07	66.82
SC70	0	90	30	4.07	22.11
	10	40	30	4.07	9.83
	20	88	30	4.07	21.62
SC57g	0	22	10	1.36	16.21
	10	39	30	4.07	9.58
	20	21	26	3.53	5.95
SC63g	0	37	30	4.07	9.09
	10	13	30	4.07	3.19
	20	31	30	4.07	7.62
SC64g	0	7	18	2.44	2.87
	10	6	21	2.85	2.11
	20	8	30	4.07	1.97

Table 3.2: Pore counts and pore densities obtained via application of the pore detector and subsequent manual validation. Pore density is calculated as n/A , where n is pore count and A is image area. Note that strain here refers to mechanical strain applied to cells during experiments.

Pore counts per image, presented in Figure 3.4, were found to be non-normally distributed for all six cell lines and conditions bar one (SC67 at 0% strain), which was borderline normally distributed (highest $p = 0.0029 > 0.0028$, One-sample Kolmogorov-Smirnov test (after Bonferroni correction)). Pore density was not significantly different from Poisson distributed for all cell lines and conditions (lowest $p = 0.0853 > 0.0028$, chi squared goodness of fit test (after Bonferroni correction)). This result is expected, given that pore density has previously been found to be non-normally distributed [8].

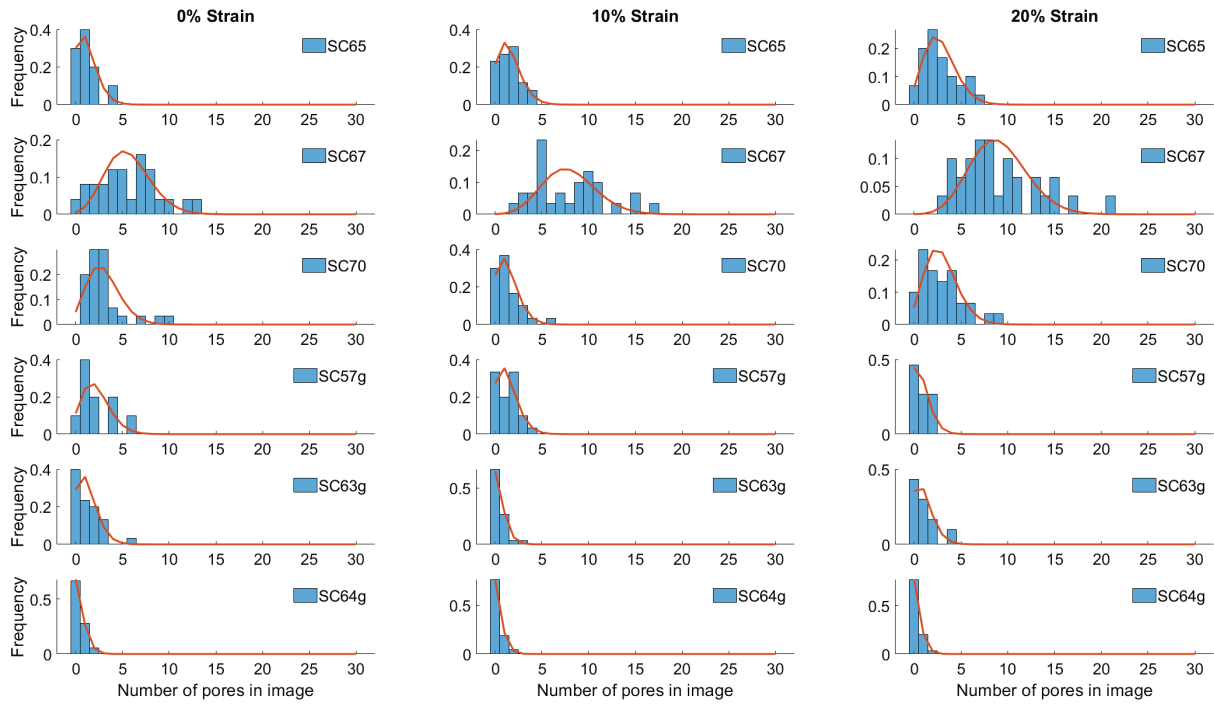


Figure 3.4: Histogram of transcellular pore count per image for all SC cell lines and experimental conditions examined. The fitted Poisson distribution of each plot is overlaid in orange.

Pore density increased significantly from 0% to 20% strain in normal SC inner wall cells from SC65 and SC67 ($p < 0.008$, E-test (after Bonferroni correction)), but not from SC70 ($p > 0.008$, E-test (after Bonferroni correction)). Pore density increased significantly from 10% to 20% strain in SC65 and SC70 cells ($p < 0.008$, E-test (after Bonferroni correction)), but not in SC67 cells ($p > 0.008$, E-test (after Bonferroni correction)). Pore density also increased significantly from 0% to 10% strain in SC67 and decreased significantly from 0% to 10% in SC70 ($p < 0.008$, E-test (after Bonferroni correction)), while no change from 0% to 10% was observed in SC65 ($p > 0.008$, E-test (after Bonferroni correction)).

Pore density in SC63g was lower at 10% strain than at 0% or 20% strain ($p < 0.008$, E test (after Bonferroni correction)). No other significant differences in pore density with stretch were observed in glaucomatous cells ($p > 0.008$, E test (after Bonferroni correction)); no increase in pore density with stretch was detected in glaucomatous cells.

Glaucomatous cell lines SC57g, SC63g and SC64g exhibited lower pore density at 20% applied mechanical strain than normal cell lines (see Table 3.2). Taking the most conservative

difference (the lowest normal density at 20% stretch and the highest glaucomatous density at 20% stretch), pore density was still significantly lower (SC63g @ 20% vs SC65 @ 20%, $p = 5.14e - 7 \ll 0.05$, E test).

3.3.2 Investigation of Relationship between Pore Measurements

To determine whether pore area, solidity and eccentricity were independent measurements, we examined plots of area versus solidity, area versus eccentricity and eccentricity versus solidity (see Figure 3.5 for example). Above roughly 5px ($0.493\mu m^2$) in pore area, there were no discernable relationships (see Figures 3.6, 3.7 and 3.8). Below 5px ($0.493\mu m^2$) in area, there appeared to be a trend towards extreme values, but this is likely due to the small area of the pores, where the location of a single pixel can affect shape measurements greatly, and so these trends are likely artefactual. As such, it is reasonable to assume that pore area, eccentricity and solidity are sufficiently independent measurements.

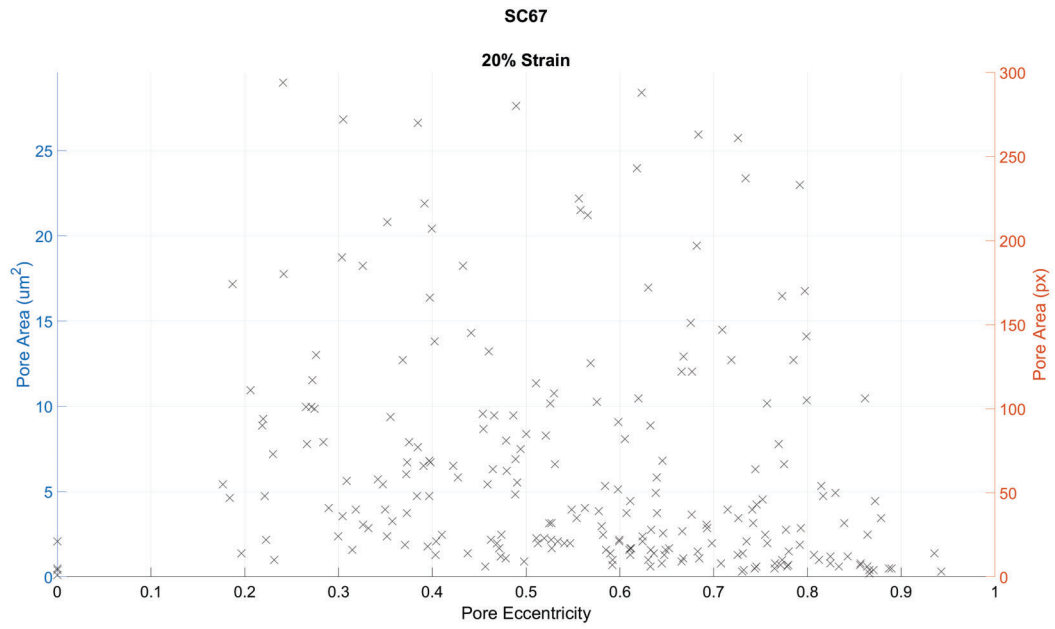


Figure 3.5: Example plot of pore area versus eccentricity. Area is shown in both square microns and pixels.

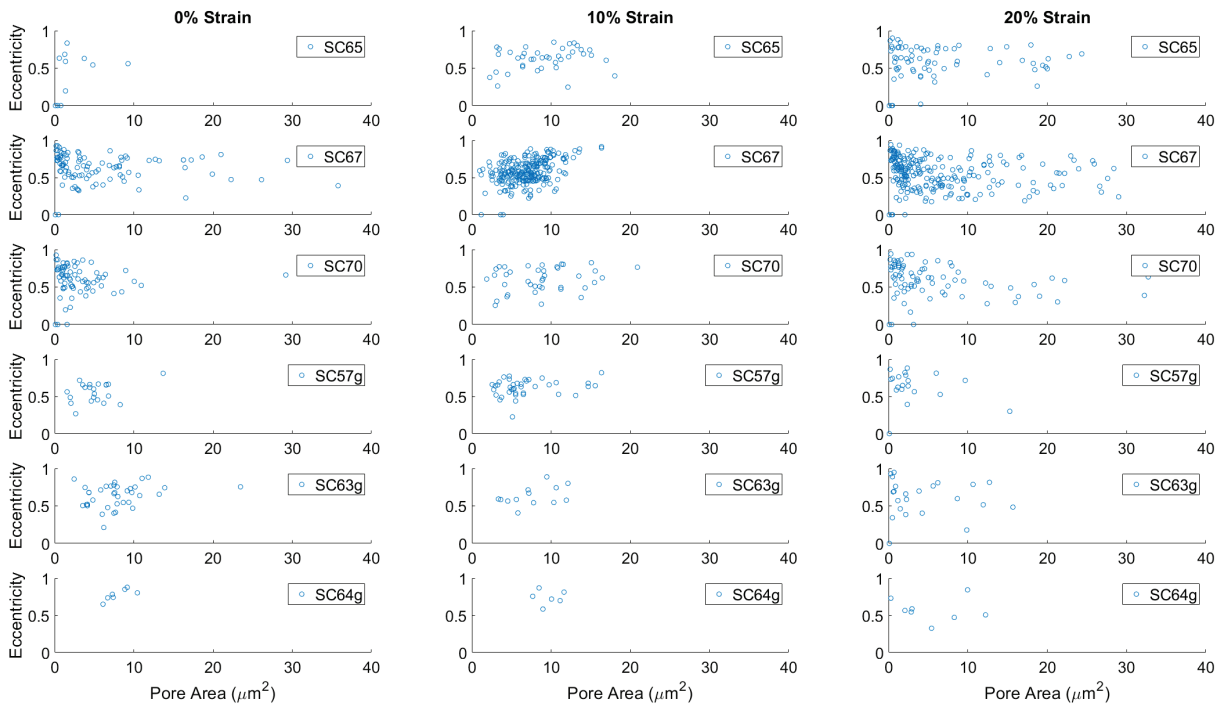


Figure 3.6: Pore area versus eccentricity, reported by experimental condition.

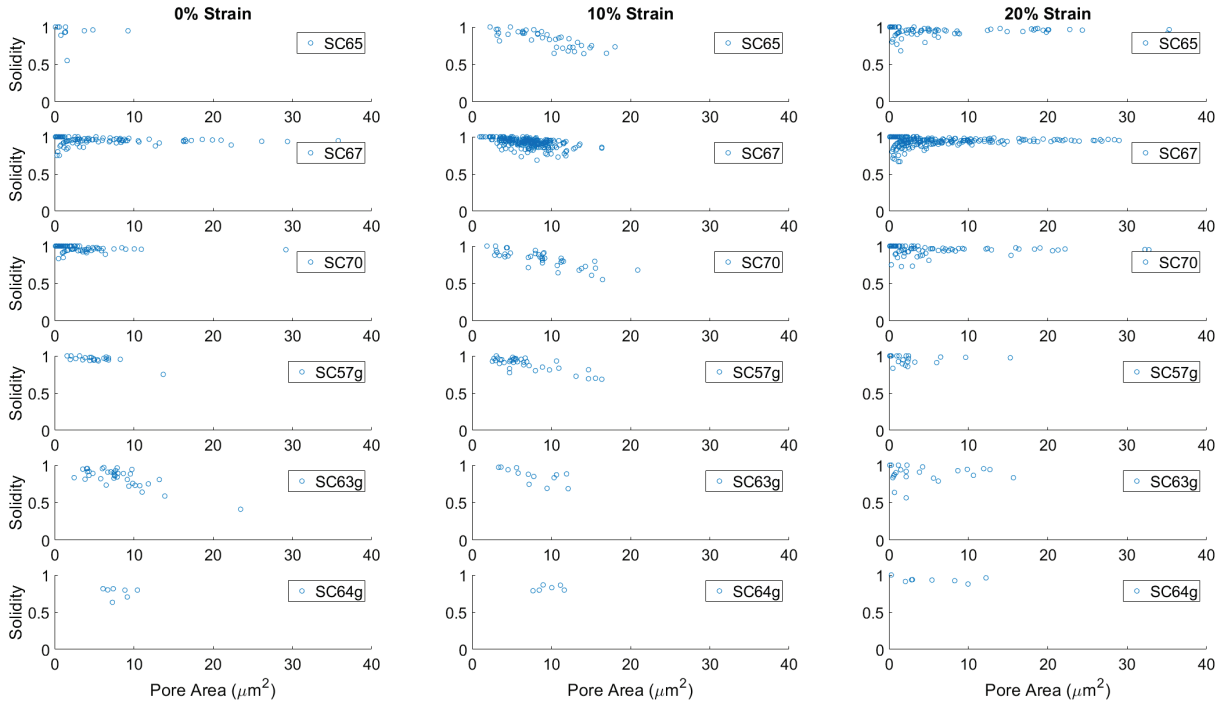


Figure 3.7: Pore area versus solidity, reported by experimental condition.

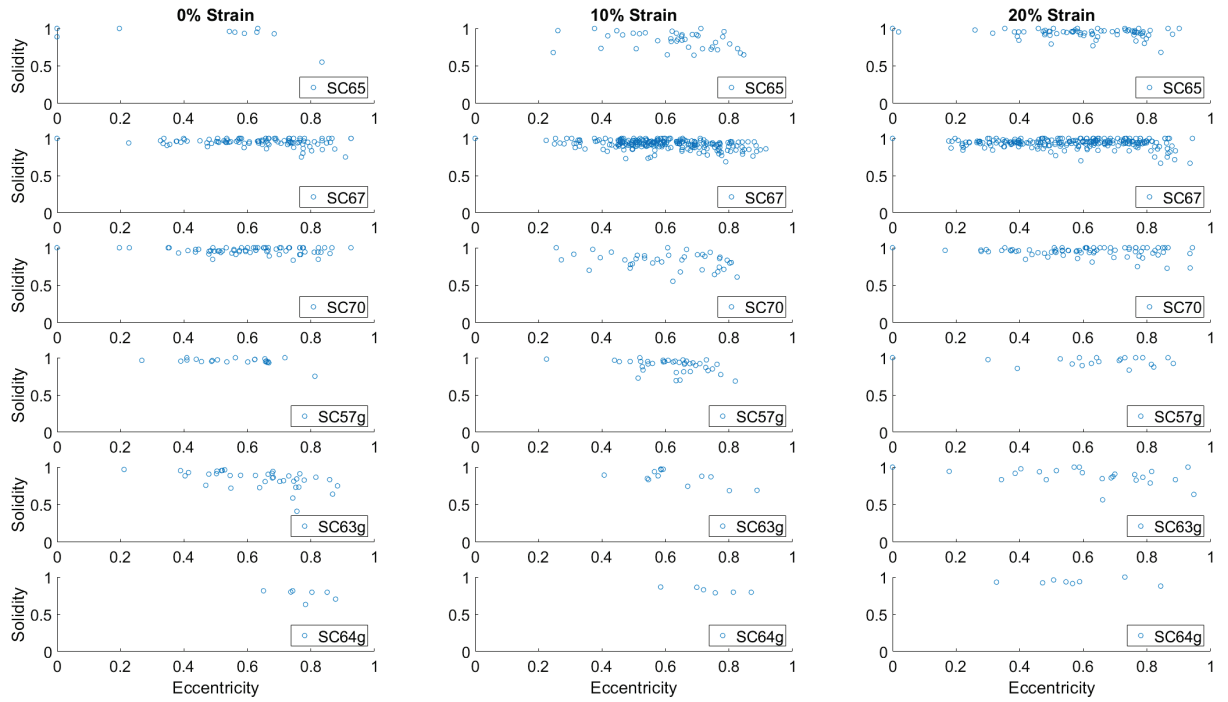


Figure 3.8: Pore eccentricity versus solidity, reported by experimental condition.

3.3.3 Pore Area Trends

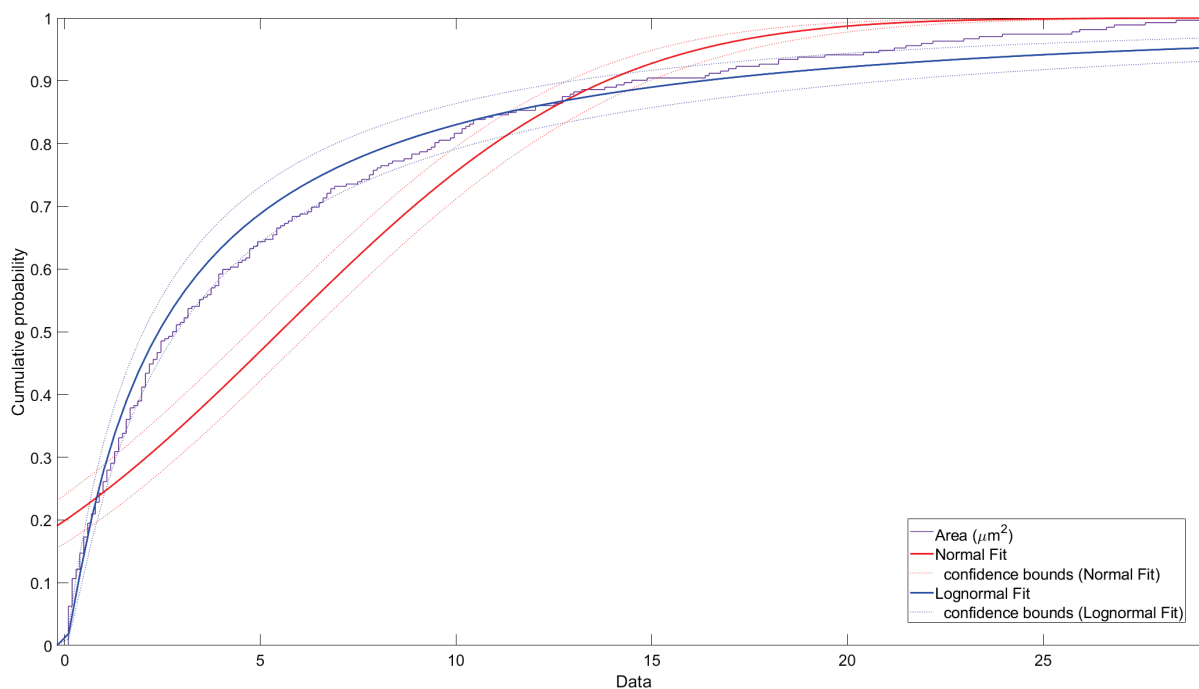


Figure 3.9: Example of pore area goodness of fit to normal and lognormal distributions.

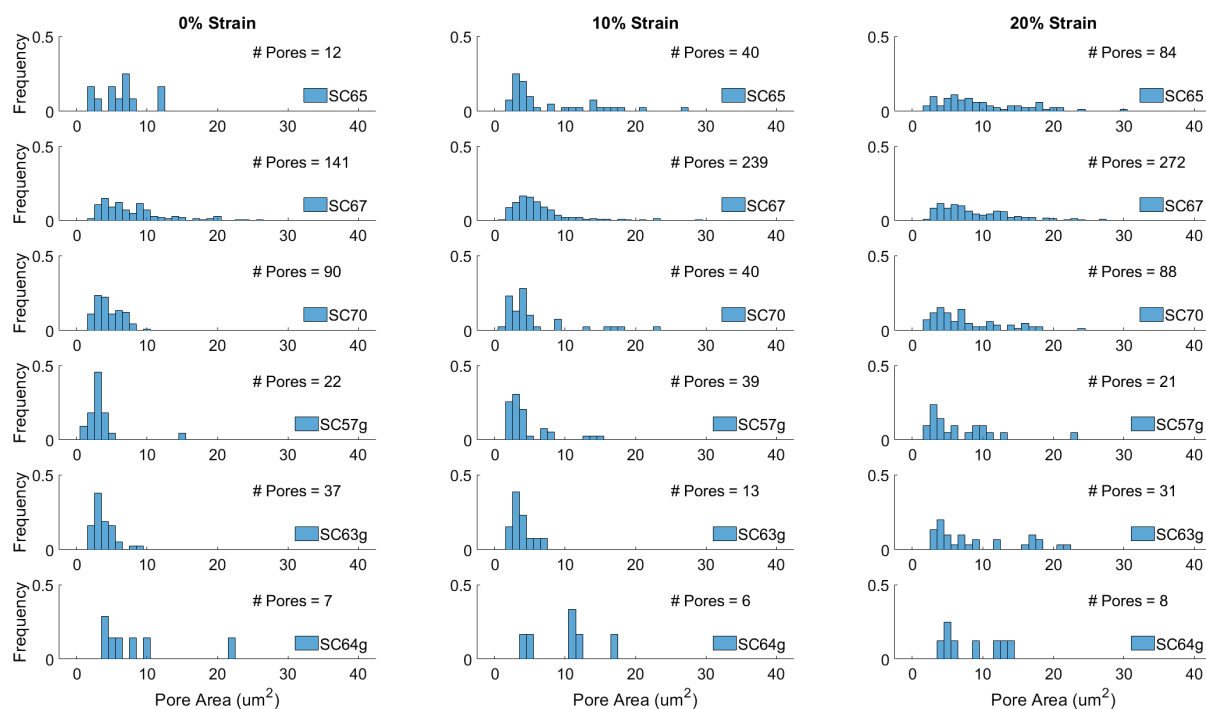


Figure 3.10: Histogram of transcellular pore area per image for all SC cell lines and experimental conditions examined. Histogram counts are displayed as relative frequencies to control for different pore numbers between conditions, allowing for fair comparison.

Pore area distribution was significantly different from normal (highest $p < 0.0028$, One-sample Kolmogorov-Smirnov test (after Bonferroni correction)) (see Figure 3.10). Considering the natural logarithm of pore area, all conditions but one (SC67 at 10% strain) were not significantly different from distributions in the normal family ($p > 0.0028$, Lilliefors test, (after Bonferroni correction)), and appeared more consistent with normal distributions (see Figure 3.9 for example). We thus performed a nested multiway analysis of variance (ANOVA) on the natural logarithm of pore area rather than the area data itself.

Nested multiway ANOVA showed a borderline effect of disease state on pore area ($p = 0.0558 \approx 0.05$ nested multiway ANOVA). Cell line (nested within disease state) and stretch (nested within cell line) both showed an effect on cell area ($p < 0.05$, nested multiway ANOVA).

When aggregated by cell line, mean pore area increased with stretch in normal cells, and in glaucomatous cells, but mean pore area was smaller in glaucomatous cells than normal cells for all stretch levels (see Figures 3.11 and 3.12). In normal cells, mean pore area was significantly higher at 20% strain ($8.78 \pm 1.20 \mu m^2$, $N = 444$) than at 0% strain ($6.29 \pm 1.72 \mu m^2$, $N = 243$) or 10% strain ($6.44 \pm 0.95 \mu m^2$, $N = 319$) ($p < 0.016$, two sample t test (after Bonferroni correction)). Mean pore area was not significantly different in normal cells between 0% and 10% strain ($p > 0.016$, two sample t test (after Bonferroni correction)), but given the data, we would have had only 80% of detecting a difference of 28% or greater.

This trend was replicated in glaucomatous mean pore area. Glaucomatous mean pore area was significantly higher at 20% strain ($8.06 \pm 1.25 \mu m^2$, $N = 60$) than at 0% strain ($5.32 \pm 2.81 \mu m^2$, $N = 66$) or 10% strain ($6.05 \pm 3.46 \mu m^2$, $N = 58$) ($p < 0.016$, two sample t test (after Bonferroni correction)). Again, no significant difference in mean pore area between 0% and 10% strain was observed in data from glaucomatous cells ($p > 0.016$, two sample t test (after Bonferroni correction)), although the smallest difference detectable with 80% power was 60% in this case.

Mean pore area was significantly larger in normal cells versus glaucomatous cells at 0% and 10% strain ($p < 0.016$, two sample t test (after Bonferroni correction)), but was not

significantly different at 20% strain ($p > 0.016$, two sample t test (after Bonferroni correction)), although there was only an 80% chance of detecting a difference of 45% or more with these data.

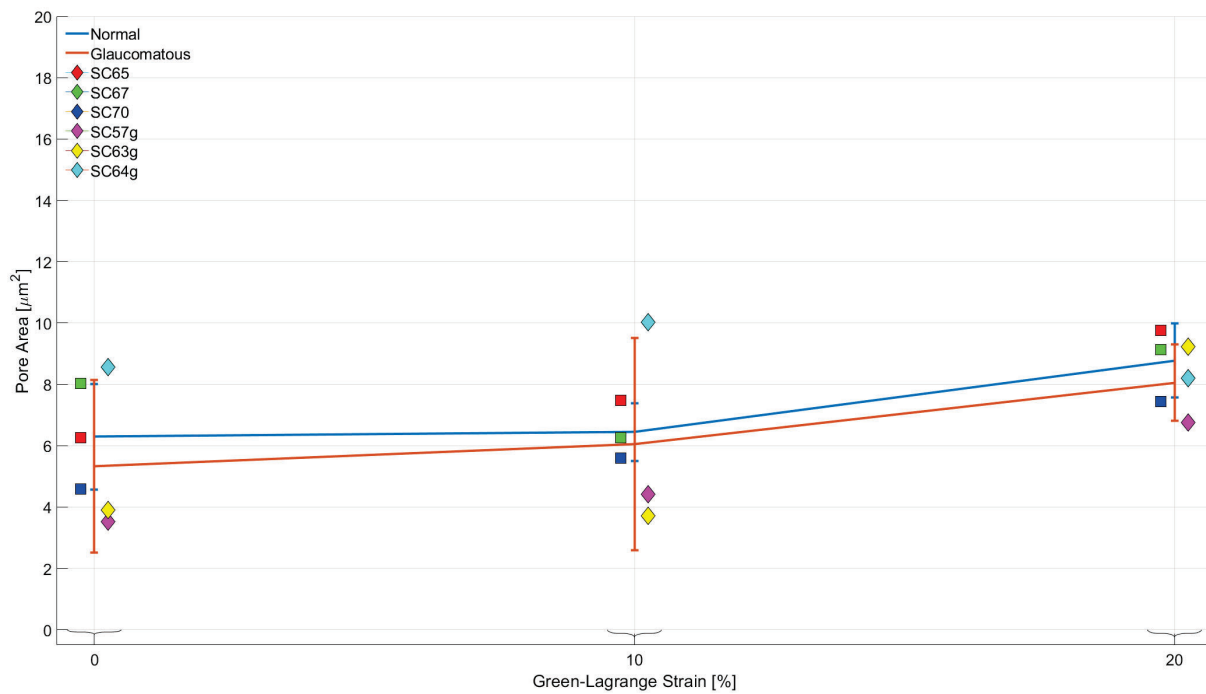


Figure 3.11: Mean pore area versus applied mechanical stretch. Line plot shows average values aggregated across cell lines, grouped by disease state. Scatter plots show means for individual cell lines. Colored squares indicate average pore areas for different normal cell lines at a given strain, while colored diamonds indicate average pore areas for different glaucomatous cell lines at a given strain.

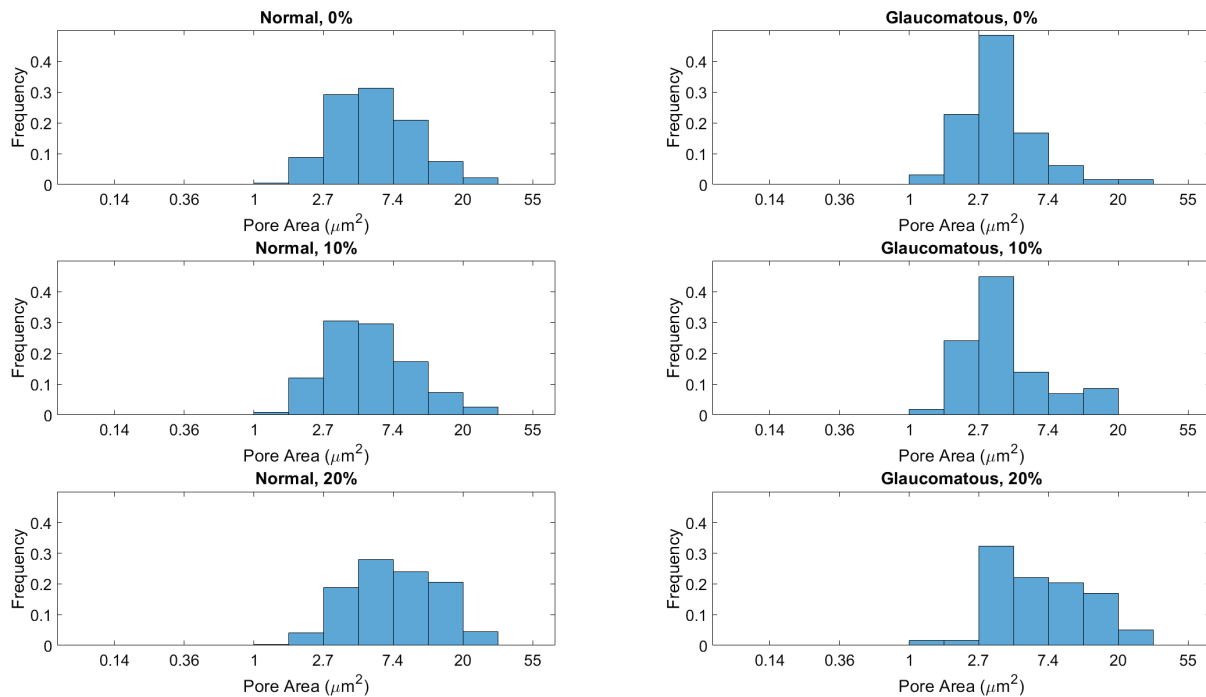


Figure 3.12: Pore area in normal and glaucomatous SC cells, grouped by cell line, at different applied mechanical strains. Histogram counts are displayed as relative frequencies to control for different pore numbers between conditions, allowing for fair comparison.

3.3.4 Pore Shape Trends

Trends in eccentricity and solidity were investigated by performing a nested multiway analysis of variance (ANOVA). There was no effect of disease state or cell line on eccentricity ($p > 0.05$, nested multiway ANOVA). There was, however, an effect of stretch on pore eccentricity ($p < 0.05$, nested multiway ANOVA).

When grouped by cell line, mean pore eccentricity increased slightly with stretch (see Figures 3.13 and 3.14). In normal cells, mean pore eccentricity decreased significantly from 0% strain (0.60 ± 0.05 , $N = 243$) to 10% strain (0.58 ± 0.01 , $N = 319$) before increasing significantly back to unstretched levels at 20% strain (0.63 ± 0.03 , $N = 444$) ($p < 0.016$, two sample t test (after Bonferroni correction)). Mean pore eccentricity was not significantly different between 0% and 20% strain ($p > 0.016$, two sample t test (after Bonferroni correction)), but given these data, there was only an 80% chance to detect a 26% difference.

In glaucomatous cells, mean pore eccentricity increased with stretch, from 0.57 ± 0.11 ($N = 66$) at 0% strain to 0.63 ± 0.04 ($N = 58$) at 10% strain to 0.64 ± 0.05 ($N = 60$) at 20% strain. Mean pore eccentricity was significantly different when strain was applied versus the undeformed state ($p < 0.016$, two sample t test (after Bonferroni correction)), but mean eccentricity was not significantly different between 10% and 20% strain in glaucomatous cells ($p > 0.016$, two sample t test (after Bonferroni correction)). However, given these data, there was only an 80% chance of detecting a difference of 60% or more.

Mean pore eccentricity was significantly higher in normal cells versus glaucomatous cells at 0% strain ($p < 0.016$, two sample t test (after Bonferroni correction)), but showed no significant difference at 10% or 20% strain ($p > 0.016$, two sample t test (after Bonferroni correction)), with an 80% chance to detect a minimum difference of 47% and 45%, respectively. It should be noted, however, that the magnitude of differences in eccentricity values is small, on the order of 10%, and so may not be physiologically relevant.

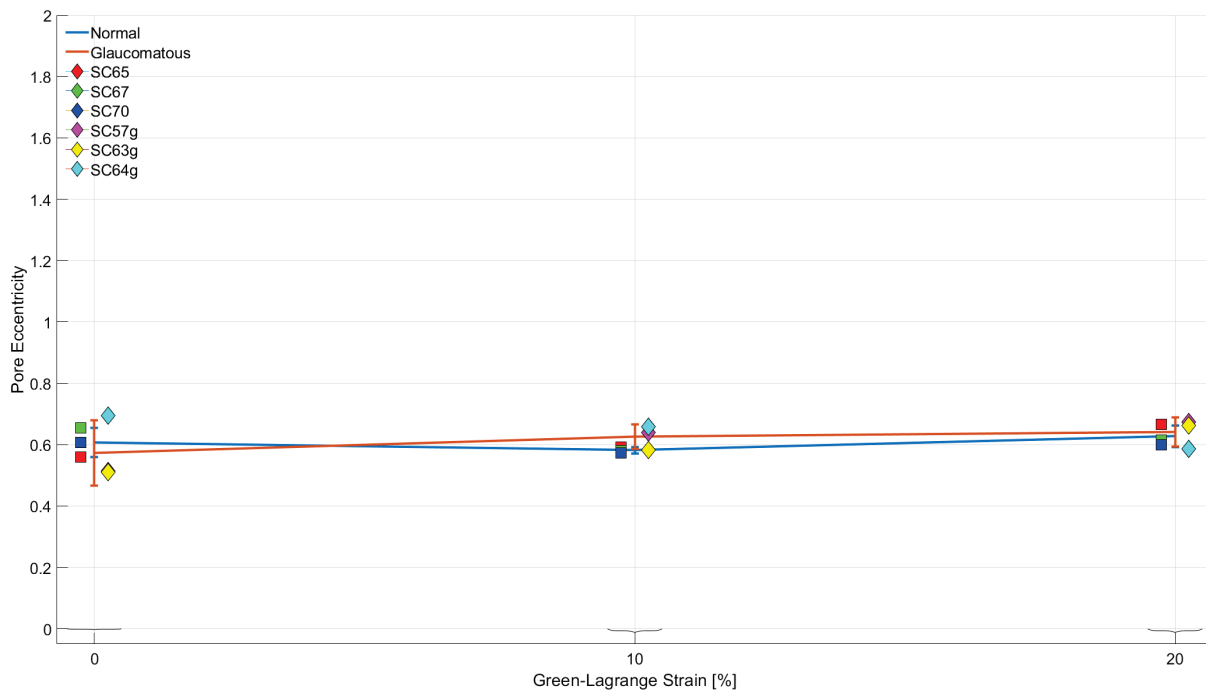


Figure 3.13: Mean pore eccentricity versus applied mechanical stretch. Line plot shows average values aggregated across cell lines, grouped by disease state. Scatter plots show means for individual cell lines. Colored squares indicate average pore areas for different normal cell lines at a given strain, while colored diamonds indicate average pore areas for different glaucomatous cell lines at a given strain.

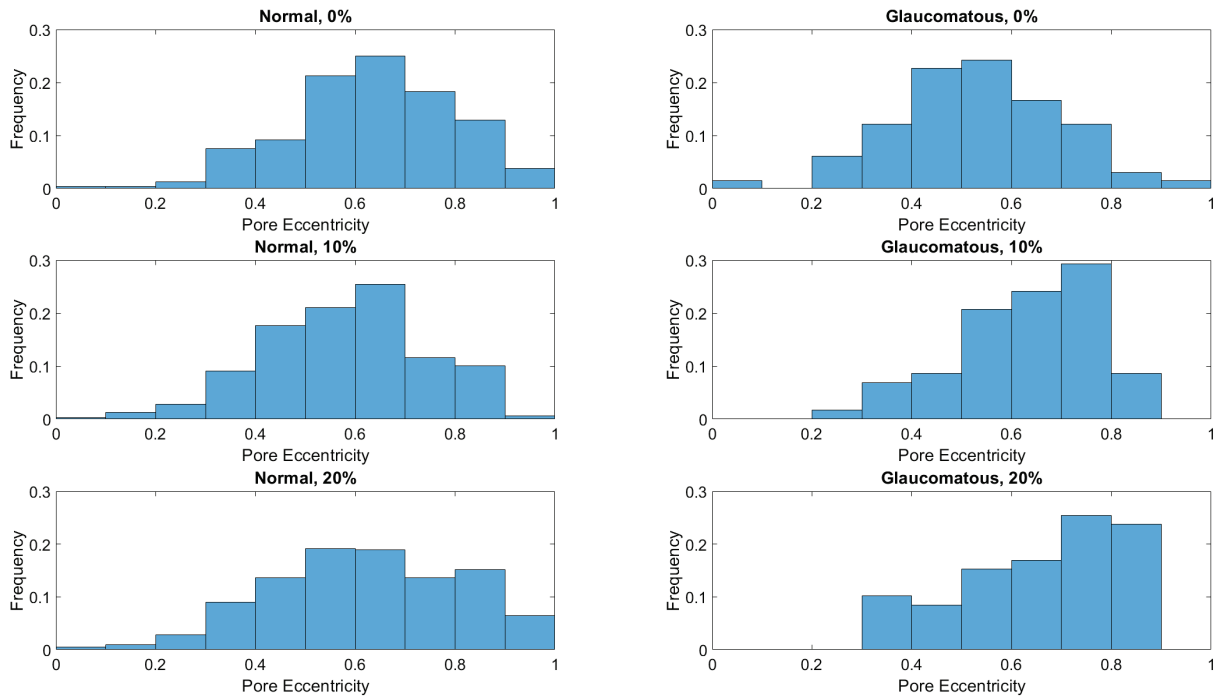


Figure 3.14: Pore eccentricity in normal and glaucomatous SC cells, grouped by cell line, at different applied mechanical strains. Histogram counts are displayed as relative frequencies to control for different pore numbers between conditions, allowing for fair comparison.

Disease state, cell line and stretch all showed an effect on pore solidity ($p < 0.05$, nested multiway ANOVA). In normal cells, mean pore solidity at 20% strain (0.88 ± 0.04 , $N = 444$) was significantly lower than at 0% (0.91 ± 0.06 , $N = 243$) or 10% strain (0.91 ± 0.03 , $N = 319$) ($p < 0.016$, two sample t test (after Bonferroni correction)) (see Figures 3.15 and 3.16). Mean solidity showed no significant difference between 0% and 10% strain in normal cells ($p > 0.016$, two sample t test (after Bonferroni correction)). No significant change in glaucomatous mean pore solidity with stretch was detected, and there was no detectable difference in mean solidity between normal and glaucomatous datasets ($p > 0.016$, two sample t test (after Bonferroni correction)). It should be noted, however, that with the data available, the lowest minimum difference detectable between conditions at 80% power was 24%. The magnitudes of differences in solidity values were again small, on the order of 5% or less, and so any differences are unlikely to be physiologically relevant.

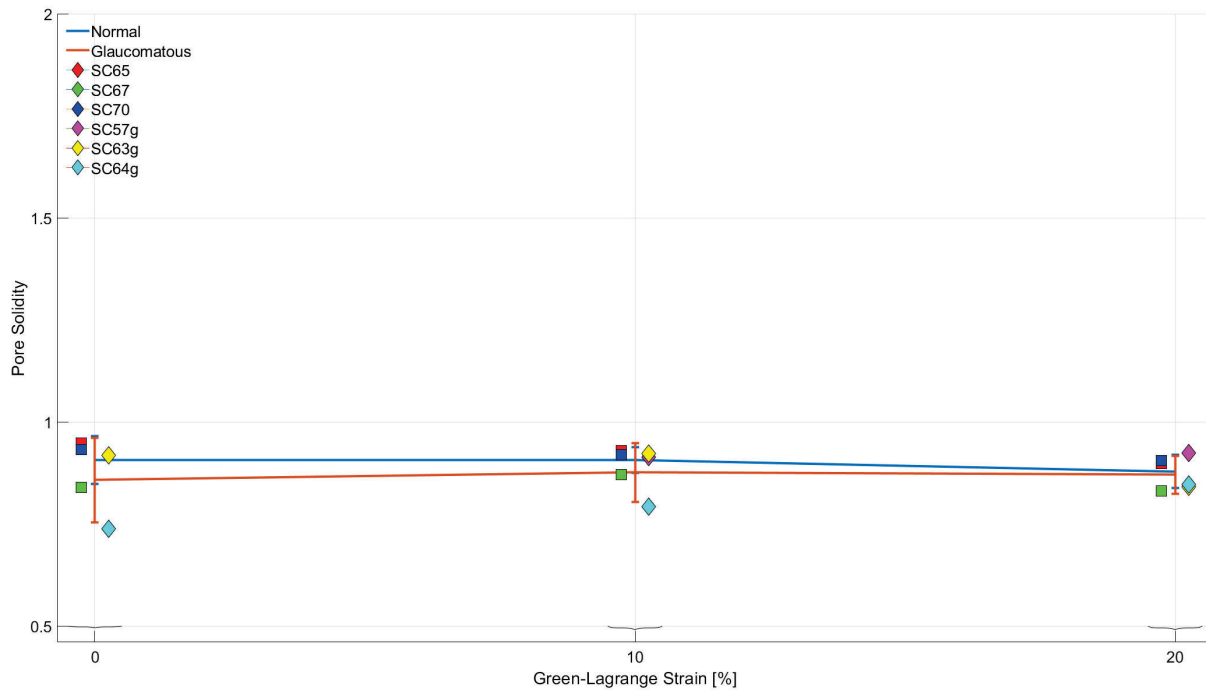


Figure 3.15: Mean pore solidity versus applied mechanical stretch. Line plot shows average values aggregated across cell lines, grouped by disease state. Scatter plots show means for individual cell lines. Colored squares indicate average pore areas for different normal cell lines at a given strain, while colored diamonds indicate average pore areas for different glaucomatous cell lines at a given strain.

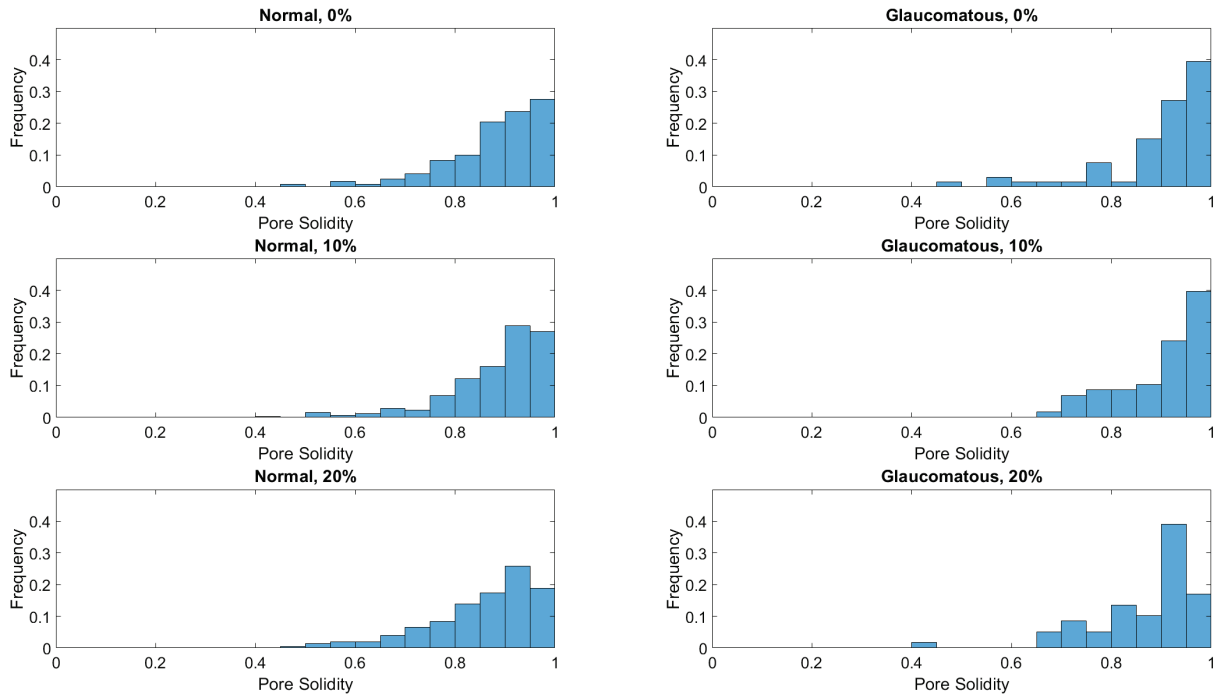


Figure 3.16: Histogram of pore solidity in normal and glaucomatous SC cells at 20% strain. Histogram counts are displayed as relative frequencies to control for different pore numbers between conditions, allowing for fair comparison.

3.3.5 Estimated Outflow Resistance due to Identified Pores

Cell Line	Strain (%)	d (μm)	" nd " (μm^{-1}), $\times 10^{-6}$	" nd^2 " $\times 10^{-6}$	" nd^3 " (μm), $\times 10^{-6}$	R ($mmHg\mu l^{-1}min^{-1}$)
SC65	0	2.60	24.02	70.50	219.53	0.870
	10	2.67	32.48	107.82	408.61	0.467
	20	3.20	66.96	247.32	989.25	0.193
SC67	0	2.94	125.63	418.54	1,511.44	0.126
	10	2.61	159.05	467.97	1,506.07	0.127
	20	3.13	215.15	765.74	2,944.68	0.065
SC70	0	2.32	51.77	127.70	327.24	0.583
	10	2.32	23.76	68.28	229.06	0.833
	20	2.81	61.41	197.67	692.41	0.276
Normal Avg.	0					0.526 $\pm$ 0.375
	10					0.476 $\pm$ 0.353
	20					0.178 $\pm$ 0.106
SC57g	0	1.95	32.81	72.53	181.28	1.053
	10	2.16	21.63	53.78	148.95	1.282
	20	2.61	16.46	51.13	177.25	1.077
SC63g	0	2.14	19.86	45.09	106.61	1.791
	10	2.12	6.86	15.11	34.20	5.582
	20	3.08	23.96	86.59	342.89	0.557
SC64g	0	3.00	9.00	31.25	120.32	1.587
	10	3.37	7.32	26.87	103.26	1.849
	20	3.06	6.19	20.51	71.17	2.682
Glau. Avg.	0					1.477 $\pm$ 0.381
	10					2.904 $\pm$ 2.336
	20					1.439 $\pm$ 1.108

Table 3.3: Moments of pore diameter and outflow resistance R estimated using Sampson's Law for various SC cell lines and strain levels. Values in bold correspond to averaged outflow resistance values across cell lines for the same strain and disease state.

The outflow resistances calculated for each experimental condition (presented in Table 3.3) were averaged across cell lines to compare between stretch and disease conditions (see bold values in Table 3.3).

Average estimated outflow resistance in normal SC cells was reduced by 66% at 20% strain versus unstretched, while average estimated outflow resistance only decreased by 3% in

glaucomatous SC cells from 0% to 20% stretch.

Averaged across cell lines, estimated outflow resistance in glaucomatous SC cells was 181% higher at 0% strain, 510% higher at 10% strain and 709% higher at 20% strain versus normal cells.

3.4 Discussion

SC inner wall pore density likely plays a key role in AH outflow resistance, since pores are the only sites where AH can cross the otherwise continuous inner wall of SC and thus drain out of the eye via the conventional outflow pathway. Previous studies have noted that SC pore density is reduced in glaucoma [10], and that SC inner wall pore formation is induced by applied mechanical strain [8]. This makes SC inner wall pore density a key focus in glaucoma research. Previous studies of pore density have used a load control model, where SC cells are cultured to confluence and perfused in the basal-to-apical direction. This is done to mimic in-vivo conditions as much as possible while still retaining a method of forming pores, typically via the perfusion pressure difference or flow rate. The findings in such studies that glaucomatous SC cells are less able to form pores cannot be decoupled from the fact that cultured glaucomatous SC cells exhibit increased subcortical stiffness compared to their normal counterparts. This motivated the shift in experimental paradigm from load control to strain control via a cell stretcher. In [1], preliminary evidence was given that pore density was reduced in glaucomatous SC cells in a strain controlled experimental paradigm, but only one glaucomatous cell line was examined. This preliminary finding is confirmed by the analysis presented here, across several cell lines over several square millimeters of examined culture area.

The data presented above agree with previously established trends; transcellular pore density increases with strain in normal cells [8], and transcellular pore density is reduced in glaucomatous cells vs. normals [10]. These findings indicate that the reduced pore density seen in glaucomatous cells must be at least partly independent of cell stiffness. However, it

should be noted that the pore densities reported here are lower than previously seen [1]. This is likely due to increased selectiveness in the pore identification process, which is in turn due to the explicitly defined selection criteria detailed in section 2.3.1. This increase in selectiveness may have led to the machine learning model or observer rejecting pores that would have been accepted previously, such as objects associated with border staining regions. Despite this, the trend seen in previous work is observed again here.

Previous studies have highlighted the link between the increased subcortical stiffness of glaucomatous SC cells and their reduced pore density. Of the six cell lines examined here, subcortical cell stiffness is known for four: one normal (SC67) and three glaucomatous (SC57g, SC63g, SC64g). The three glaucomatous cell lines studied here are on average 2.1x stiffer than SC67 (Prof. Mark Johnson, Northwestern University, personal communication), while pore density is on average 7x higher in normal cell lines at 20% stretch. However, when investigating the relationship between subcortical stiffness and pore density in individual cell lines, no correlation was observed. This is somewhat intuitive, given that these findings are obtained using a deformation controlled scheme and so the influence of cell stiffness should be minimized in these data. Despite this, it has not been confirmed whether the strain applied to the substrate is entirely transferred to the cultured cells as one would expect; stiffer cells may resist stretching by partially releasing from the deforming substrate, or glaucomatous SC cells may be less adherent to the culture substrate and thus partially release when stretched, experiencing a smaller strain than expected and thereby producing fewer pores. An investigation into how SC cells deform when their substrate deforms should be conducted to confirm whether the cells in fact experience the strains we apply to the substrate.

The results presented above indicate that pore density was not affected by stretch in glaucomatous cells. Not only did we not detect an increase, the data suggested that SC pore density appeared to decrease with stretch, although this effect was not statistically significant. There may be several possible explanations for this result, one of which is that the glaucomatous cells were not, in fact, being deformed to the same degree as their normal counterparts, perhaps due to their elevated subcortical stiffness, or because they may have partially detached from the substrate. This again highlights the need to investigate whether

substrate deformation is transmitted to normal and glaucomatous cells to the same degree, as discussed immediately above. It is also possible that glaucomatous cells are less sensitive to stretch than normal cells, to the point that a 20% equibiaxial strain is not sufficient to induce a significant change in pore formation. Using data from [47], it has been shown in [27] and [18] that inner wall cells in ocular hypertensive rhesus monkeys underwent estimated areal strains per cell due to giant vacuole formation of up to 228%, which would correspond to equibiaxial strain of up to 50%, while the maximum equibiaxial strain applied in this study was 20%, falling short of the full physiological range theoretically possible for these cells. Because larger deformations occurred at IOP above 21mmHg and elevated IOP is related to glaucoma, it is possible that glaucomatous SC cells may require a greater deformation than their normal counterparts to trigger a significant increase in pore density. This finding may thus argue for the need for a wider range of strain levels to be applied in future experiments.

The mechanisms specific to SC pore formation are still unknown. However, it is theorized that SC pore formation may function similarly to pore formation in other endothelia [27]. If this theory is correct, then pore formation should require the fusion of the SC cell's basal and apical membranes, which typically requires fusogenic proteins. Thus, one possible explanation for the decreased pore density observed in glaucomatous SC cells for the same mechanical strain could be decreased expression and/or production of fusogenic proteins such as VAMP2 and 3. It is known that glaucomatous cells display increased stiffness in the cytoskeleton, and while the findings presented above suggest that the glaucomatous pore density reduction is at least partly decoupled from cell stiffness, the root cause of the two observations may still be linked. There may exist in glaucomatous cells some additional component of the cytoplasm or cytoskeleton that causes an increase in internal stiffness but also obstructs membrane fusion, essentially "getting in the way" of pore formation. Alternatively, there could be alterations in ion channel function that reduces their ability to properly transduce stretch. Another possible cause could be alterations of plasma membrane properties that inhibit pore formation. Any of these reasons could explain the observed reduction in transcellular pore density in glaucomatous SC cells, and should be investigated further.

Pore area was measured in this study, while others have reported diameter. Comparison is

possible with such studies by assuming pores to be circular and converting diameter to area accordingly. A recent SEM study of normal SC pores reported an average transcellular pore diameter of $0.41\mu\text{m}$, observing no changes in pore diameter with strain [8]. This corresponds to an average pore area of $0.13\mu\text{m}^2$, which is much smaller than the areas measured here. Average transcellular pore size, as measured in situ in perfused non-glaucomatous human eyes [48], was larger than reported in [8], but still smaller than the values presented here (diameter = $0.89\mu\text{m}$, area = $0.62\mu\text{m}^2$). This is likely due to lateral diffusion of the stain under the cell after passing through the pore in the assay used in this work. Higher molecular weight stains could be considered to counteract this. Regardless, because identical stains and stain exposure times are used between experiments, the pore area results can be compared between experimental conditions in this study.

The findings presented above indicate that pore area increases with strain. This contradicts findings in a previous study, which detected no change [8]. These findings cannot be explained by the increase in area due to applying stretch to the cells, since all stretcher membranes were imaged in the undeformed state. Because SEM studies typically examine such a small cell area and because pores are such rare structures, it is possible that this trend could have been missed due to the small number of transcellular pores examined in SEM studies. There is also the issue of the artefacts introduced by fixation that may be mistaken for pores during SEM analysis. It has been suggested that these artefacts may represent a large proportion of the pore-like objects seen in SEM pore studies [10], and if the size of these artefacts does not vary with stretch, said artefacts may obscure any actual trends in pore size in such studies.

Because the fluorescent assay labels pores before fixation, this issue is avoided, which may explain why a strain dependent area increase is seen here but was not seen in SEM studies.

Pore area is on average lower in glaucomatous SC cells for the same applied strain, which is, to the best of our knowledge, a novel finding, although this decrease was only significant at 0% and 10% strain. Using the area data presented above and using Sampson's Law to estimate the outflow resistance attributable to an inner wall with the observed distribution of pores, we find that outflow resistance decreases less with stretch in glaucomatous SC cells vs their normal counterparts. Additionally, while the difference in pore area is small, it results in

between 181% and 709% increased resistance versus normal cells for the same applied mechanical strain. If these differences are replicated *in vivo*, it would suggest that the increase in outflow resistance seen in glaucoma would be attributable not solely to pore density as previously thought, but rather to total pore area.

The calculated outflow resistance values presented here are higher than those calculated by Bill and Svedbergh in [6]. This difference can be explained by the differences in pore density and pore diameter observed in the data presented above versus the SEM studies of Bill and Svedbergh. The fluorescent pore staining technique used here produces pores with diameters 2.7 times larger than SEM pores, on average, which would result in a 20 ($= 2.7^3$) fold reduction in outflow resistance. However, the pore densities observed here are 100 fold lower than in [6]. The combined effect of these differences with respect to Bill and Svedbergh would produce outflow resistances that are 5 times higher than the classic Bill and Svedbergh results. As Bill and Svedbergh predicted that observed pores could account for at most 10% of total outflow resistance, the factor of 5 difference would place our results within 50% of total outflow resistance, which is reasonable in comparison to a total human outflow resistance of 3 mmHg/ μ l/min.

There are several factors that should be noted when attempting to compare the calculated outflow resistance values to those seen *in vivo*. It should be noted that the pore sizes presented above are likely inflated due to the lateral diffusion of the fluorescent stain. It is also possible that some transcellular pores may be missed with the assay or during processing, and the fact that paracellular pores cannot be captured using this assay. It is also not clear that 20% equibiaxial strain is reflective of what is experienced by the inner wall *in situ*. Additionally, it is also unclear whether culture-induced changes in the cells (e.g., cell area) influence comparisons between *in vitro* estimates of outflow resistance to *in vivo* measurements. We thus stress that the outflow resistance values are meant for the purpose of comparison within the results obtained using this assay, to illustrate the possible effect that the observed differences in pore area can cause.

Pore eccentricity and solidity showed some significant changes with stretch and disease state.

However, it should be stressed that the actual changes in measured eccentricity and solidity was minor, and both glaucomatous and normal eccentricity and solidity distributions were highly consistent across experimental conditions. These observed changes are thus unlikely to have significant physiological relevance.

One possible explanation for the observed differences in pore shape metrics could be the capture of the pore during closure. Previous SEM studies have observed membranous extensions, reaching across the openings of pores [8], and there are similarities between these extensions and the ventral lamellipodia observed in closure of small wounds in vascular endothelial cells [49]. If this trend is replicated in vivo, these structures may obstruct AH flow, depending on their stiffness. Upon manual inspection of fluorescent assay pores with low solidity and/or low eccentricity, no conclusive evidence of these structures could be seen, but this may be due to the low resolution of pores in the image data. Naturally, the ability to discern such fine structures is limited by the resolution of the image data. It also seems unlikely that such structures would be detectable using the fluorescent assay for pore detection, as the staining duration is likely long enough to allow the stain to diffuse under any partial obstructions.

Chapter 4

Unfinished Work: Mapping Intracellular Strain Fields and Investigating the Time-Dependence of Pore Density in SC Cells

4.1 Introduction

SC pore formation is likely an important factor in outflow resistance modulation, but little is known about SC pore formation besides the fact that SC pores are induced by cellular deformation [8] and that pore density is reduced in glaucomatous SC cells [10],[11]. The findings in Chapter 3 suggest that other aspects of SC pore formation may affect outflow resistance, such as pore area, which appears to increase with stretch and is lower in glaucomatous SC cells. This indicates that we still lack a complete understanding of how SC pores modulate outflow resistance.

Because SC pore formation is a mechanosensitive process, it is important to understand how SC cells experience deformation. A method of investigating the strain distribution within the

cell as it is deformed may provide insight into the structures responsible for the mechanotransduction of pore formation, providing new targets for SC pore research and glaucoma therapeutics. Strain experienced by the membrane may be involved in opening of stretch-activated ion channels, while strain experienced by the cytoskeleton may indicate where the cell experiences the most thinning, which may coincide with the location of pores. Such studies may also reveal what factors may limit strain-dependent pore formation in glaucomatous SC cells. To this end, we present an algorithm capable of calculating the strain field in a series of images or video using fiducial markers such as fluorescent particles that can be attached to cultured cells to enable cell tracking.

A second topic of major interest in SC pore formation is the transient nature of pores in SC cells. To investigate this, we present an experimental protocol, based upon the fluorescent pore assay developed in [1], that allows for investigation of the transient properties of SC pores. Using this new protocol, we present preliminary findings showing that pore formation is not immediately triggered at the onset of stretch but rather can occur at a delayed time after stretch is applied. We also show preliminary evidence that pores may close while the cell is still deformed. These findings indicate that SC pore density varies over time even while SC cells are exposed to a constant mechanical strain. As the pore density at any time is influenced by the rate of pore formation and the rate of pore closure, both of which may depend on stretch, it is important to begin to investigate these rates in SC cells and to examine whether there are any differences in these rates between normal and glaucomatous SC cells.

These aspects of the thesis were in progress, but were severely disrupted due to the COVID-19 pandemic and thus could not be completed in time for submission. The aspects of these works that were completed in time are presented below.

4.2 An Algorithm to Track Mechanical Strain in Cultured Cells

4.2.1 Introduction

To drain from the eye, AH passes through micron-sized pores in the inner wall endothelium of SC [9]. SC pores are reduced in glaucoma [10],[11], which may contribute to increased AH outflow resistance and elevated IOP.

SC inner wall endothelial cells experience basal-to-apical perfusion due to AH outflow, which results in a pressure gradient across the largely impermeable cell monolayer [18] between the basal and apical monolayer surfaces. This pressure gradient causes SC inner wall endothelial cells to regularly experience large deformations as they form giant vacuole structures [18]. These deformations are thought to be necessary for SC inner wall endothelial cells to form pores, since a requirement for transcellular pore formation is the thinning of the cytoplasm such that the cell's basal and apical membranes are in close proximity, facilitating fusion [27]. The ability of a cell to resist large deformations can be characterized by its stiffness, and interestingly (but perhaps not surprisingly), SC inner wall endothelial cells obtained from glaucomatous donor eyes were found to have increased subcortical stiffness relative to their normal counterparts when cultured in the same conditions [20]. It is also known that applied mechanical strain is sufficient to trigger pore formation [8]. This makes studying SC cells experiencing deformations relevant for glaucoma research.

The pressure drop across SC cells due to AH flow causes SC cells to deform, and this deformation is thought to trigger pore formation. However, the results in Chapter 3 show that even when controlling for cellular deformation (by controlling substrate stretch), glaucomatous SC cells form fewer pores than normal cells. One possible explanation for this difference in pore density may be that the glaucomatous SC cells experienced lower strain than the normal cells, for instance due to cellular detachment from the substrate. Hence, there is a need for the ability to measure cellular strain during stretch application to confirm

whether strain is effectively transmitted from substrate to cell. Additionally, it is currently unknown whether all major components of the cell (eg. cytoskeleton and plasma membrane) experience the same strain when the cells are stretched. This may provide insight into the mechanisms involved in pore formation, since pore formation is a mechanosensitive process [8]. To facilitate the investigation of these questions, a method of determining the strain field in cells is needed.

Options exist for strain tracking, either commercially or through open source libraries. However, incorporating different standalone softwares into a single pipeline, as would be desired for colocalization studies and/or use in a high-throughput screening platform, can raise compatibility issues. There is also the concern that commercial software may become deprecated and unsupported, and that open source can be unstable and there is no guarantee of long term compatibility. For these reasons, we present a custom software system for calculating the strain field experienced by cells as they are deformed, by tracking fiducial markers through frames of video data of the cells being stretched.

4.2.2 Methods

Algorithm Design

The strain tracking pipeline operates on a series of sequential frames, taking the first frame as the "reference" or "origin" state. The algorithm is designed detect and track the in-image-plane trajectories of a set of fiducial markers in order to estimate the 2D strain field in each frame, relative to this reference state. Any motion of fiducial markers normal to the image plane (i.e. "into" or "out of" the image) is ignored. Point tracking was chosen over DIC, a commonly used technique for calculating strain fields by measuring the deformation of textures rather than discrete points, for this application. Both techniques were tested initially, but point tracking was found to produce more precise and accurate results in initial testing. This, combined with the reduced complexity of the technique, resulted in the strain tracking algorithm being developed as a point tracking based algorithm.

To simplify point registration and displacement calculations, all frames are first registered to the first frame using SIFT [34], a computer vision algorithm commonly used for image recognition and registration. Each frame is first processed with the SIFT algorithm, which detects hundreds of robust key points in each image. These key points are then registered between each pair of subsequent frames, and the most likely rigid body deformation that describes the movement of the key points in each pair of frames is calculated. The calculated deformation matrices are then inverted and applied to each frame to undo any shifting of points in the plane of the image due to unintended movements of the camera and specimen relative to each other. This helps mitigate issues with point tracking introduced due to the field of view shifting relative to the reference state. This module is most important when parts of the experimental setup must be moved by hand during the experiment, as when using manually actuated cell stretchers.

After registration, fiducial markers in the images are detected. These fiducial markers can be anything detectable in the images via thresholding, but for the intended application of cellular strain tracking will typically be fluorescent microspheres or other similar fluorophores which can be directly attached to cells. Each frame is first converted to grayscale. An adaptive threshold is then applied to mask out fiducial markers by keeping pixels which are sufficiently distinct from the local background according to a manually set sensitivity threshold.

Adaptive thresholding [44] aids in identifying markers that may have lower contrast due to uneven illumination in the frame. 8-connected components labelling is then performed, and the centroids of all objects found are returned. Objects of insufficiently large area are neglected, as these tend to correspond to shot noise due to uneven illumination, or discontinuous fiducial marker edges, especially at high strain levels.

Once the locations of the tracked points are known in each frame, points are registered between frames. This is done using the freely available Blair & Dufresne adaptation of John C. Crocker's particle tracking algorithm [50]. This process is performed on each pair of subsequent frames. For a given pair of subsequent frames, the positions of all fiducial markers within each frame are collected. Each fiducial marker in the first frame is assigned a single corresponding fiducial marker in the subsequent frame, and the total squared displacement of

all fiducial markers is then calculated using these assigned correspondences. This process is repeated and assignments are shifted in order to minimize the total squared displacement of fiducial markers, where the minimum total squared displacement is assumed to correspond to the correct assignment.

This algorithm would require $O(N!)$ operations to assign relationships to N points in two frames, which quickly becomes impractical as N increases. To address this, a limit to the distance a particle can be considered to have moved L is set. When L is set to be larger than the maximum distance moved by particles between frames and less than the typical spacing between fiducial markers, the problem of assigning relationships is broken into a set of smaller networks which can be optimally resolved separately. In the most trivial case, where L is set such that each of N points has only one possible match, the number of operations is reduced to $O(N \ln N)$. This process is then repeated for the next subsequent pair of frames, until points have been registered through all frames. The result is a series of trajectories for each particle in the origin frame.

Once point registration has been performed, neighbouring triplets of points are grouped together, so that the strain calculation can be performed. Registered points are grouped into triplets via Delaunay triangulation [51]. Briefly, this algorithm takes a set of points and produces a set of triangles with those points as vertices, where the circumcircle associated with each triangle contains no other point in its interior.

Once the triangulations have been performed, the point triplets are passed to the final module, which calculates the 2D strain in each triplet relative to the corresponding triplet in the origin frame. SC inner wall experiments involving the application of stretch typically aim to apply equibiaxial strain [1], since equibiaxial strain has no directionality and so there is no need to control for cell "alignment" relative to the direction of stretch. When strain is equibiaxial, the two normal strain components, E_{xx} and E_{yy} must be equal, and the shear strain E_{xy} must be zero. When tracking equibiaxial strain, the strain tensor can thus be expected to contain three components; E_{xx} , E_{yy} and E_{xy} . Thus, sets of three points, such as the triplets of points grouped via Delaunay triangulation in the previous step, are sufficient to

fully determine the strain tensor in this case.

The strain calculation module solves for the components of the strain tensor as follows. We begin by considering an object in Cartesian space. Two points can be chosen within this object, and the vector between them can be represented by:

$$d\vec{X} = dX\hat{x} + dY\hat{y} \quad (4.1)$$

Following an applied deformation to this object, the vector between these two points in the new deformed configuration can be represented as:

$$d\vec{x} = dx\hat{x} + dy\hat{y} \quad (4.2)$$

The Green-Lagrange strain tensor E , is defined via the following relationship:

$$d\vec{x}^2 - d\vec{X}^2 = 2 \cdot d\vec{X} \cdot E \cdot d\vec{X} \quad (4.3)$$

This formulation can be expanded to yield a single equation with three unknown variables, namely the three components of E :

$$\begin{bmatrix} E_{xx} & E_{xy} \\ E_{xy} & E_{yy} \end{bmatrix} \quad (4.4)$$

where E_{xx} and E_{yy} are the normal strains in the x and y directions, respectively, and E_{xy} is the shear strain. The resulting expansion yields:

$$d\vec{x}^2 - d\vec{X}^2 = 2dX^2E_{xx} + 4dXdY E_{xy} + 2dY^2E_{yy} \quad (4.5)$$

Because this equation has three unknowns, at least three such equations, for three different

vectors (and their deformed counterparts), must be solved simultaneously. The resulting set of simultaneous equations, each of which is derived in the same manner, can be expressed in matrix form as:

$$M \cdot x = b \quad (4.6)$$

Where:

$$M = \begin{bmatrix} 2dX_1^2 & 4dX_1Y_1 & 2dY_1^2 \\ 2dX_2^2 & 4dX_2Y_2 & 2dY_2^2 \\ 2dX_3^2 & 4dX_3Y_3 & 2dY_3^2 \end{bmatrix} \quad (4.7)$$

$$x = \begin{bmatrix} E_{xx} \\ E_{xy} \\ E_{yy} \end{bmatrix} \quad (4.8)$$

$$b = \begin{bmatrix} d\vec{x}_1^2 - d\vec{X}_1^2 \\ d\vec{x}_2^2 - d\vec{X}_2^2 \\ d\vec{x}_3^2 - d\vec{X}_3^2 \end{bmatrix} \quad (4.9)$$

where n in $d\vec{x}_n$ and $d\vec{X}_n$ refers to point n in the deformed and undeformed triplet, respectively. To solve this set of equations, we first left multiply both sides by M^T , (i.e. the transpose of M), which yields:

$$M^T \cdot M \cdot x = M^T \cdot b \quad (4.10)$$

Next, we left multiply both sides of the equation by $(M^T \cdot M)^{-1}$ (i.e. the inverse of $M^T \cdot M$), which results in the solution for x :

$$x = (M^T \cdot M)^{-1} \cdot M^T \cdot b \quad (4.11)$$

where $(M^T \cdot M)^{-1} \cdot M^T = M^+$ (i.e. the left Moore-Penrose pseudoinverse of M).

The algorithm assigns the values of E calculated for a given triplet to the centroid of that triplet. As such, the algorithm outputs the calculated strain field in an image as a point-field, with each point centered within a triplet of points.

Algorithm Validation

The strain tracker was tested using the twist-actuated cell stretcher detailed in section 2.2.4 (see Figure 2.3). Briefly, the stretcher design consists of a set of three concentric rings; the outermost ring, or "screwtop", the intermediate ring, or "membrane holder", and the inner ring, or "indenter ring". The screw-top and the membrane holder are threaded, so that twisting the screw-top pulls the membrane holder up, towards the screw-top. The "membrane holder" clamps the stretcher membrane, a thin sheet of PDMS where cells are seeded, into the system and holds it in place with an o-ring as the indenter pushes down on it. When the threads are engaged but the system is at zero turns, the innermost ring, or indenter, sits flush against the inside face of the screw-top's lip, and flush with the bottom face of the membrane holder, contacting but not deforming the stretcher membrane. As the screw-top is turned, the membrane holder rises, pulling the membrane against the indenter ring, applying an equibiaxial strain to the membrane. A pin inserted into a slot between the indenter and membrane holder ensures the indenter does not rotate relative to the membrane holder, thereby ensuring the membrane does not experience shear stress, enforcing the equibiaxial condition. Silicone lubricant is applied to the indenter ring to ensure low friction contact between the indenter ring and the stretcher membrane, ensuring the membrane slides smoothly and uniformly over the indenter ring and thus that the strain field is applied uniformly across the stretcher membrane.

To test the strain tracker with the twist actuated cell stretcher, hexagonal arrays of points

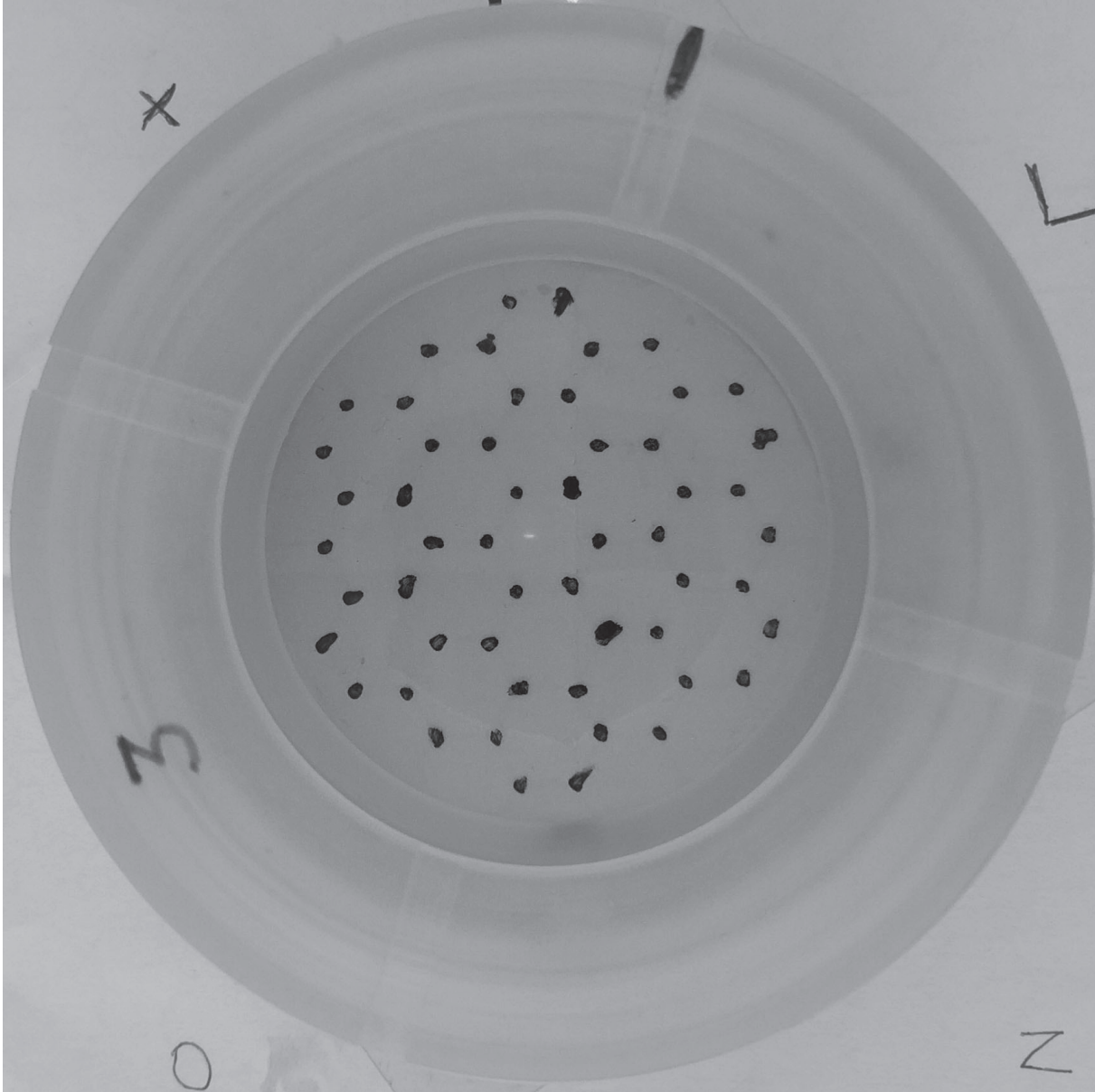


Figure 4.1: Example of hexagonal point grid used for calibration of the twist actuated cell stretcher.

were drawn on the stretcher membranes of three stretchers using a stencil grid (see Figure 4.1 for example). The expected strain applied by the stretchers was determined analytically based on the design parameters of the threads, which is derived by calculating the expected deformation of the stretcher membrane for a given number of rotations using the geometry of the stretcher design, as follows (see Figure 4.2).

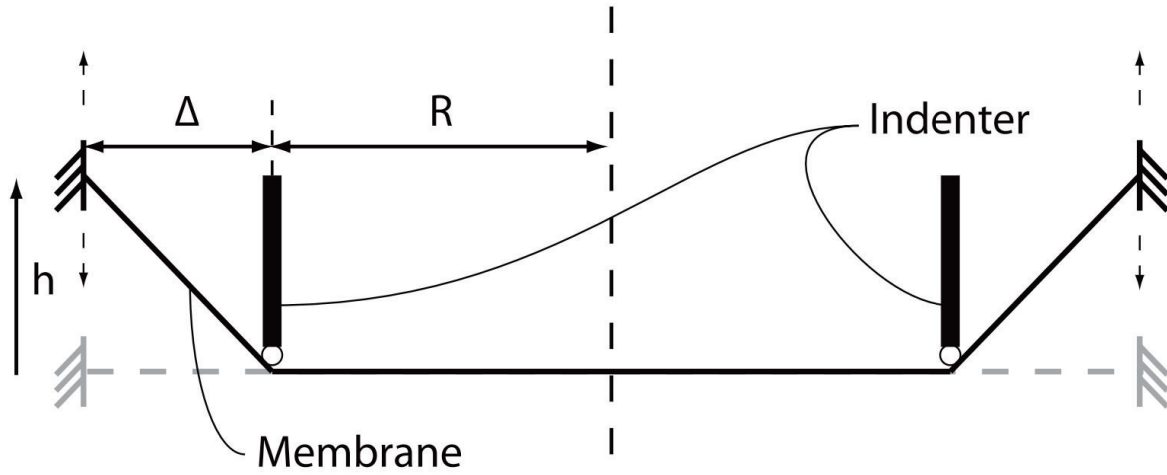


Figure 4.2: Diagram depicting the geometry of the twist actuated stretcher when the membrane is stretched. The center dashed line denotes the central axis of the cylindrical stretcher. Reproduced from [1].

The twist actuated cell stretcher's membrane produces equibiaxial strain, and the stretcher itself is cylindrical, making the problem axisymmetric. Let R denote the radius of the indenter ring, and let Δ denote the distance between the intermediate ring and the indenter ring. The membrane in the undeformed state thus has radius:

$$R + \Delta \quad (4.12)$$

in the undeformed state from the clamping point to the central axis of the stretcher. If h is defined as the depth to which the indenter ring is pushed down by actuating the stretcher, the length of the membrane between these same two points is:

$$R + \sqrt{h^2 + \Delta^2} \quad (4.13)$$

Where h is determined by the number of turns n and the pitch of the stretcher threads θ according to:

$$h = n\theta \quad (4.14)$$

The stretch ratio, λ , can thus be written as the ratio of equations 4.13 and 4.12:

$$\lambda = \frac{R + \sqrt{h^2 + \Delta^2}}{R + \Delta} \quad (4.15)$$

The Green-Lagrange strain, ϵ_{GL} , is then defined in terms of λ as:

$$\epsilon_{GL} = \frac{\lambda^2 - 1}{2} \quad (4.16)$$

Three stretchers of identical design were used, with three membranes each. Each membrane was subjected to a range of applied mechanical strain from 0% to 40% in increments of 5% and imaged at each step. These images were then passed to the strain tracker for analysis. The strain as calculated by the strain tracker was then compared to the expected analytical strain.

Once the accuracy of the strain tracker had been assessed, we next tested its ability to track fiducial markers in microscopy images using preliminary experimental data obtained by Jacques Bertrand. Tracking was tested using a PDMS substrate micropatterned with $75\mu m$ diameter circular islands of biotinylated gelatin and stained with FITC-avidin (Jacques Bertrand, personal communication) (see Figure 4.3).

In addition, $1\mu m$ diameter fluorescent microspheres (FluoSpheresTM Sulfate Microspheres, 1.0 μm , yellow-green fluorescent (505/515), 2% solids, F8852) were applied to the stretcher membrane in a random pattern by incubating a solution of microspheres on the membrane

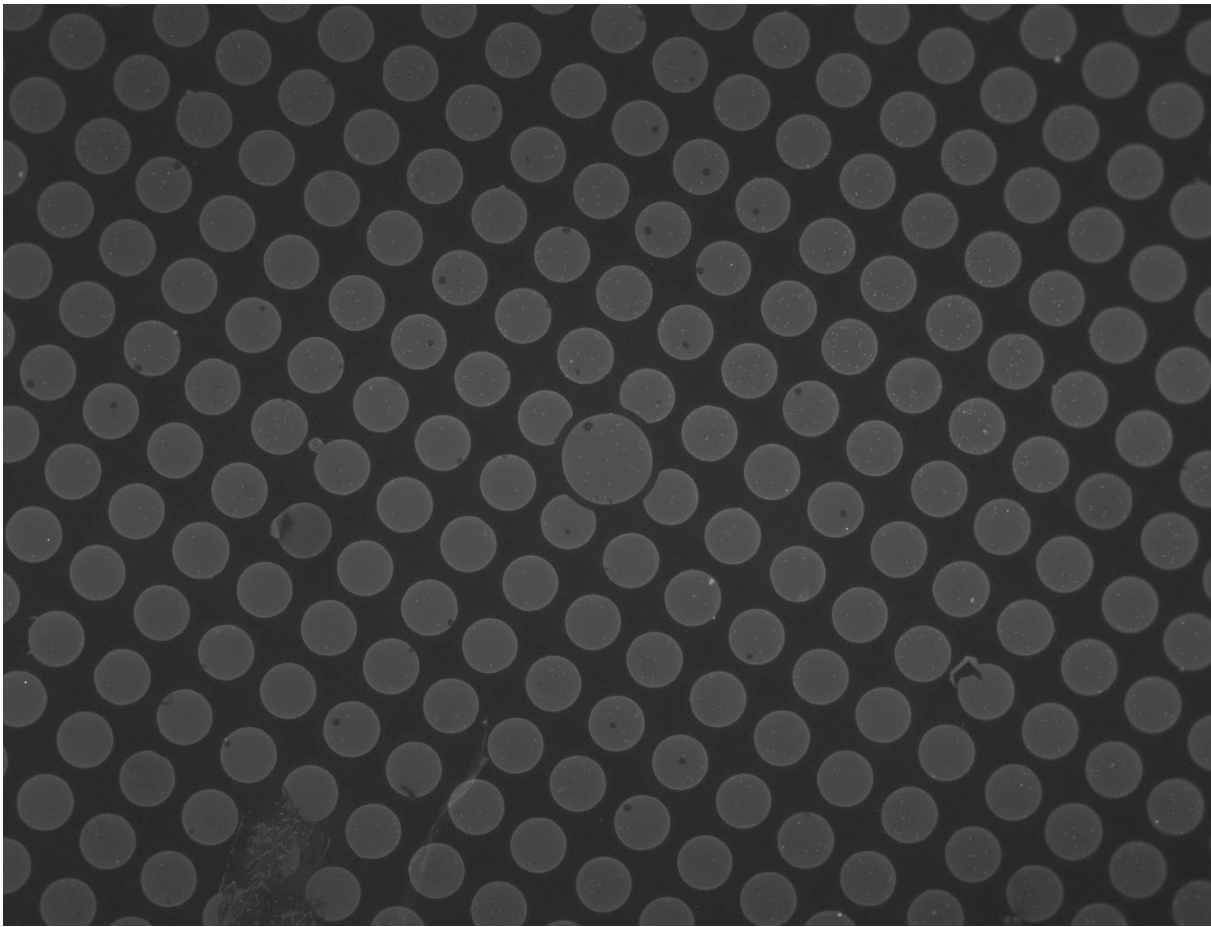


Figure 4.3: Example of micropatterned substrate used for testing strain tracking algorithm at microscopic scale.

surface. The area surrounding the micropatterned islands was PEGylated to prevent attachment of microspheres to areas other than the islands, resulting in a grid of clusters of microspheres to be tracked.

The micropatterned, microsphere coated membrane was then placed in a prototype vacuum actuated cell stretcher for video microscopy based cell deformation studies. The strain field of this prototype stretcher was independently characterized and validated (Jacques Bertrand, personal communication). The stretcher was placed on the stage of a fluorescent microscope and the membrane was imaged at 20x as the stretcher was actuated. The experiment began in the undeformed state at $t=0$ s. Strain was applied at $t = 0.05$ s, ramping from 0% strain at $t = 0.05$ s to 15% equibiaxial strain at $t = 0.25$ s, which was maintained for the remainder of the video. The strain at each frame of video captured was calculated by the strain tracker to

determine if the points were adequately tracked. The micropatterned substrate was imaged before, at $t = 0$ s, and after application of 15% equibiaxial strain, at $t = 0.5$ s.

4.2.3 Results

Performance on Twist Actuated Stretcher Calibration Patterns

In all experiments the strain reported by the strain tracker was equibiaxial. The average strain calculated by the strain tracker was similar to the analytical solution, although in some experiments the calculated strain began to differ above 3 turns (see Figures 4.4, 4.5 and 4.6). In stretcher 3, the average strain plateaued at 3.5 turns rather than continuing to increase linearly as expected.

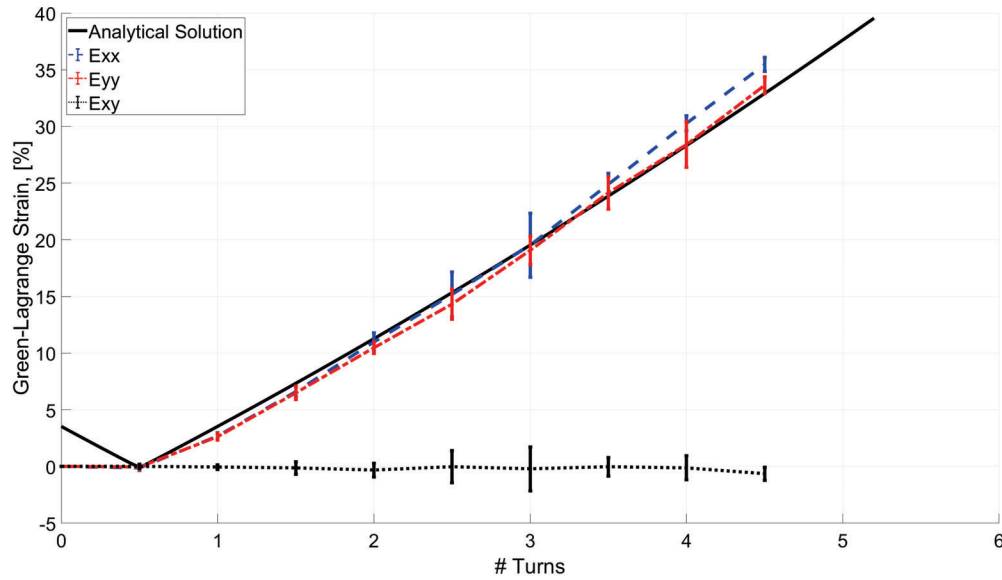


Figure 4.4: Average strain field estimation at each frame of hand drawn fiducial markers in a series of images for stretcher 1.

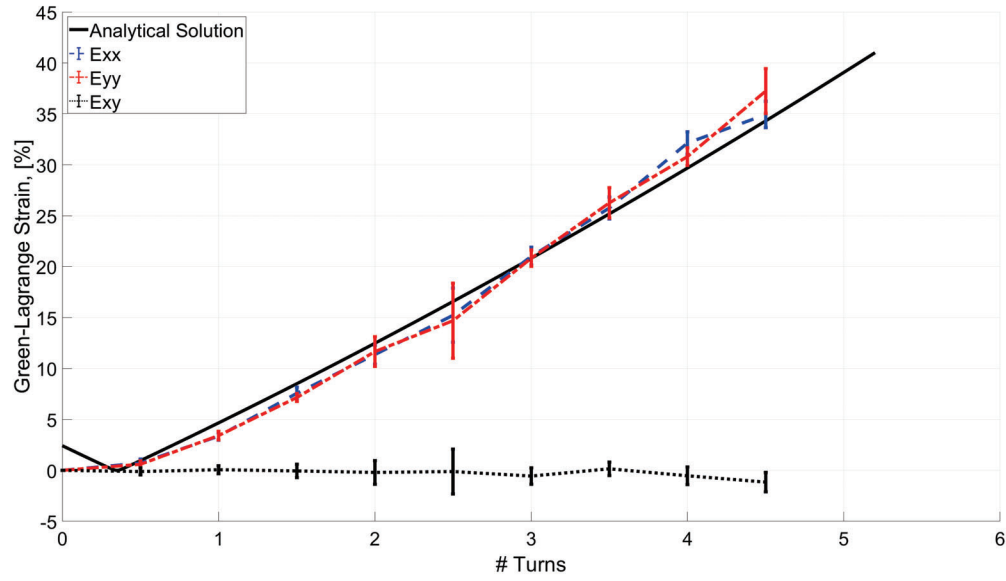


Figure 4.5: Average strain field estimation at each frame of hand drawn fiducial markers in a series of images for stretcher 2.

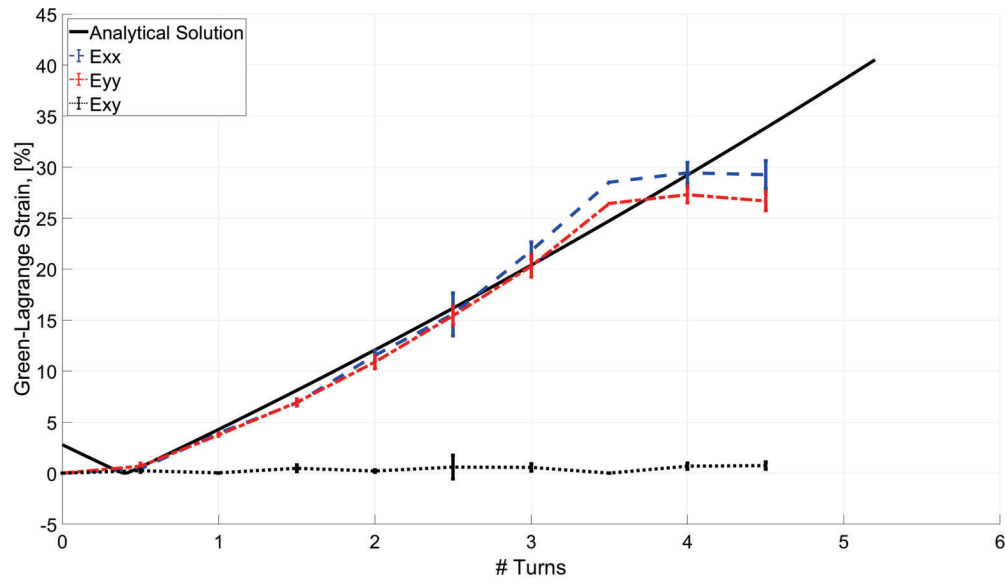


Figure 4.6: Average strain field estimation at each frame of hand drawn fiducial markers in a series of images for stretcher 3.

Performance on Microscopy Image Data of Micropatterned Substrate

The strain tracker reported the expected overall strain field expected from the design of the prototype cell stretcher (see Figure 4.7). Some point dropout occurred, but this mostly appeared to be caused by points leaving the tracked field of view (see Figures 4.8).

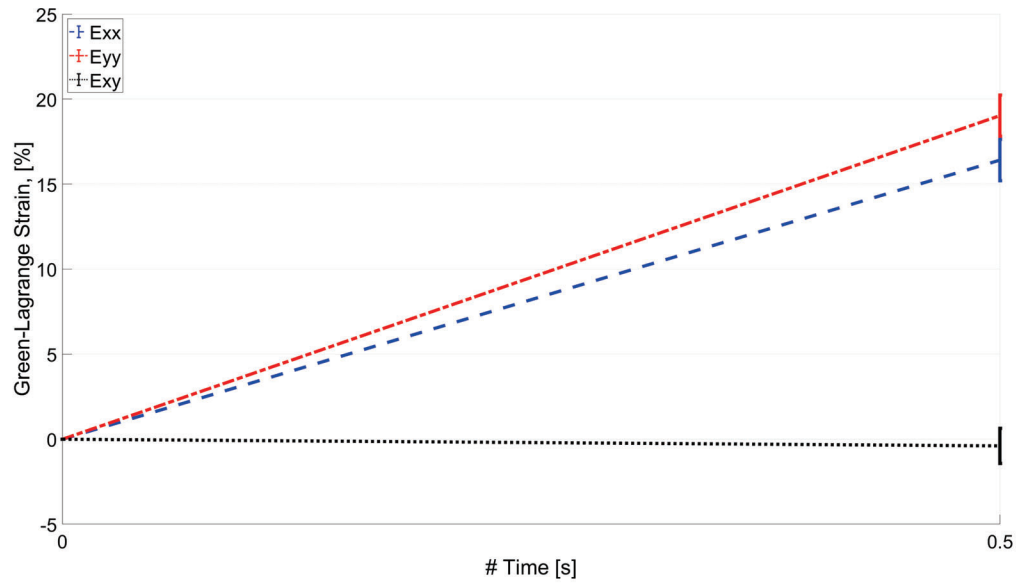


Figure 4.7: Average strain estimated before and after strain application to micropatterned substrate.

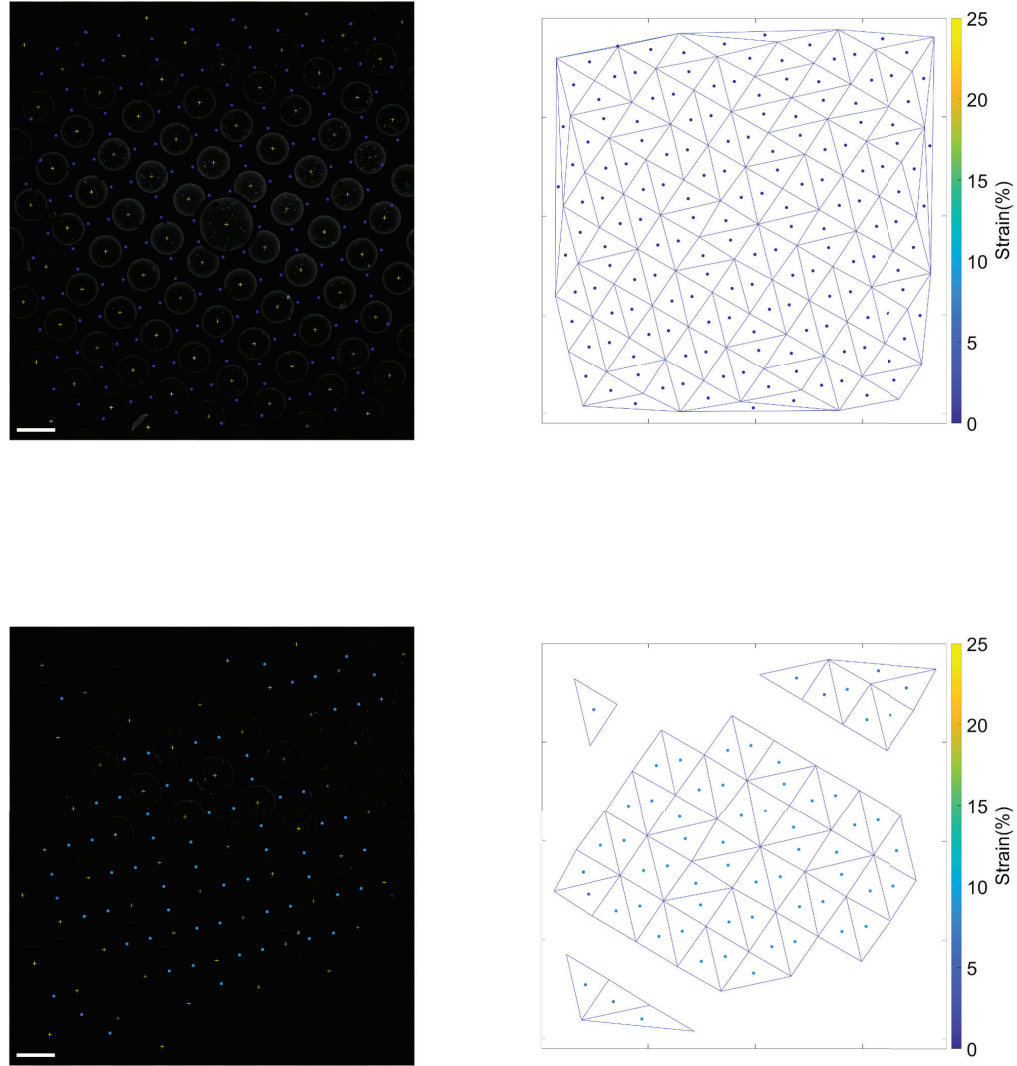


Figure 4.8: E_{xx} component of the strain field before (top panel, corresponding to frame 0) and during strain application (bottom panel, corresponding to frame 1) in micropatterned substrate images. Each colored point represents the center of a triplet of tracked points. Line segments denote the members of each Delaunay triangulation associated with each colored point. Point color indicates E_{xx} strain magnitude calculated for that triplet of points. Colorbar indicates relationship between color and strain. Scalebar $\approx 125 \mu m$.

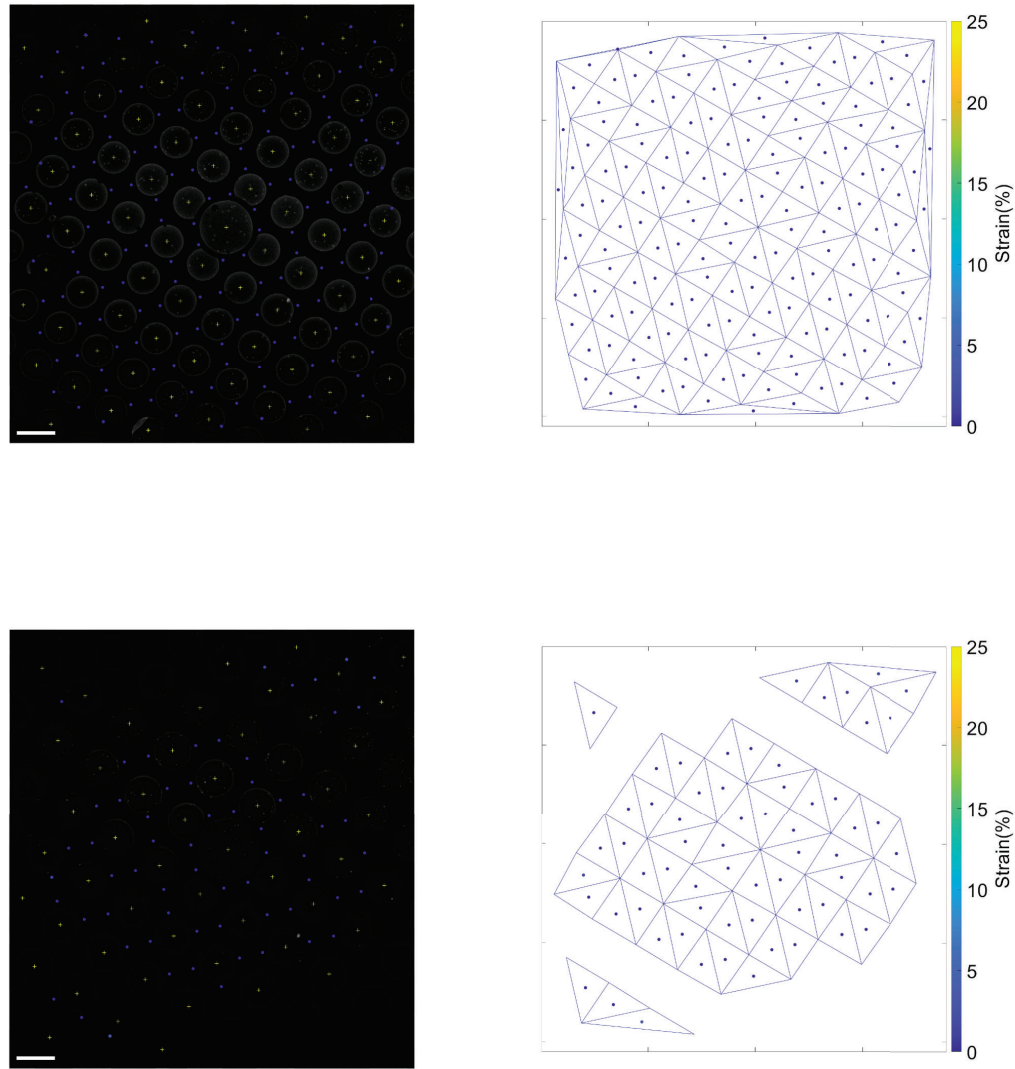


Figure 4.9: E_{xy} component of the strain field before (top panel, corresponding to frame 0) and during strain application (bottom panel, corresponding to frame 1) in micropatterned substrate images. Each colored point represents the center of a triplet of tracked points. Line segments denote the members of each Delaunay triangulation associated with each colored point. Point color indicates E_{xy} strain magnitude calculated for that triplet of points. Colorbar indicates relationship between color and strain. Scalebar $\approx 125 \mu m$.

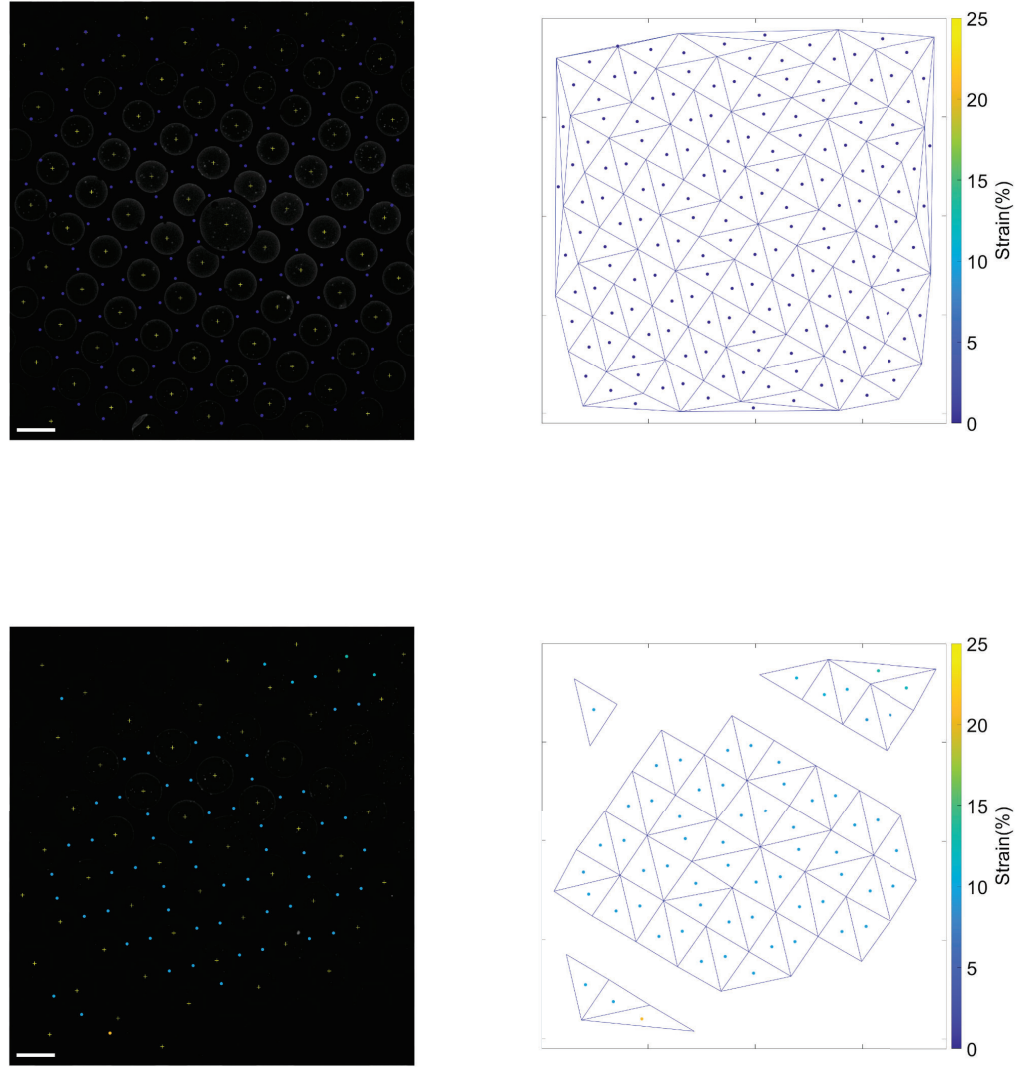


Figure 4.10: E_{yy} component of the strain field before (top panel, corresponding to frame 0) and during strain application (bottom panel, corresponding to frame 1) in micropatterned substrate images. Each colored point represents the center of a triplet of tracked points. Line segments denote the members of each Delaunay triangulation associated with each colored point. Point color indicates E_{yy} strain magnitude calculated for that triplet of points. Colorbar indicates relationship between color and strain. Scalebar $\approx 125 \mu m$.

Performance on Microscopy Video Data of Fluorescent Particles

The strain tracker again reported the expected overall strain field based on the design of the prototype cell stretcher (see Figure 4.11). Upon inspection of the strain field reported for individual frames, a large amount of point dropout was observed. The number of points was sufficient to quantify an overall strain field over the interrogated region, but large parts of the frame were no longer tracked, significantly reducing the resolution of the strain field being calculated and causing tracking to fail in those parts of the frame (see Figures 4.12, 4.13 and 4.14 for examples).

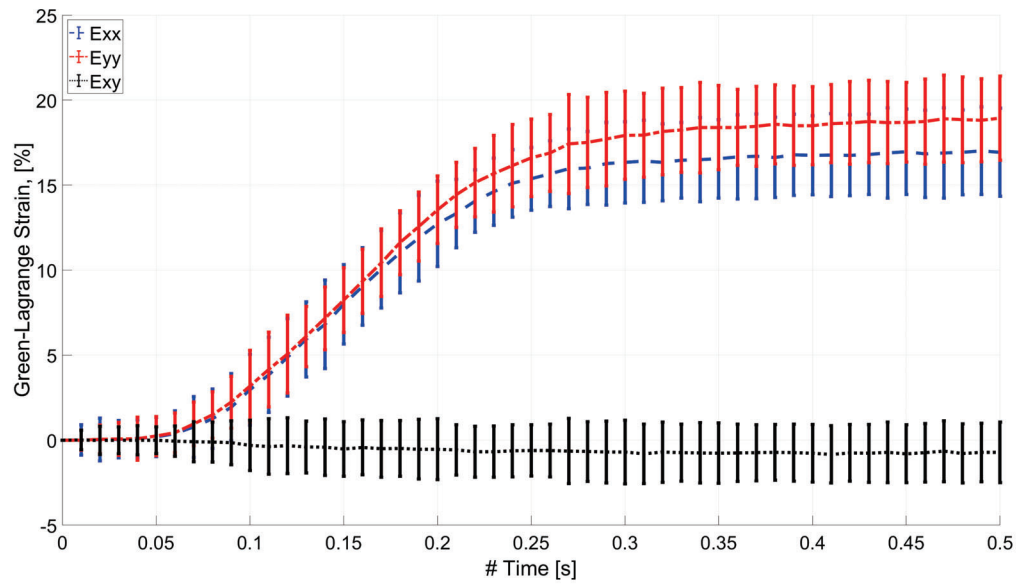


Figure 4.11: Average strain estimated at each frame of fluorescent marker video.

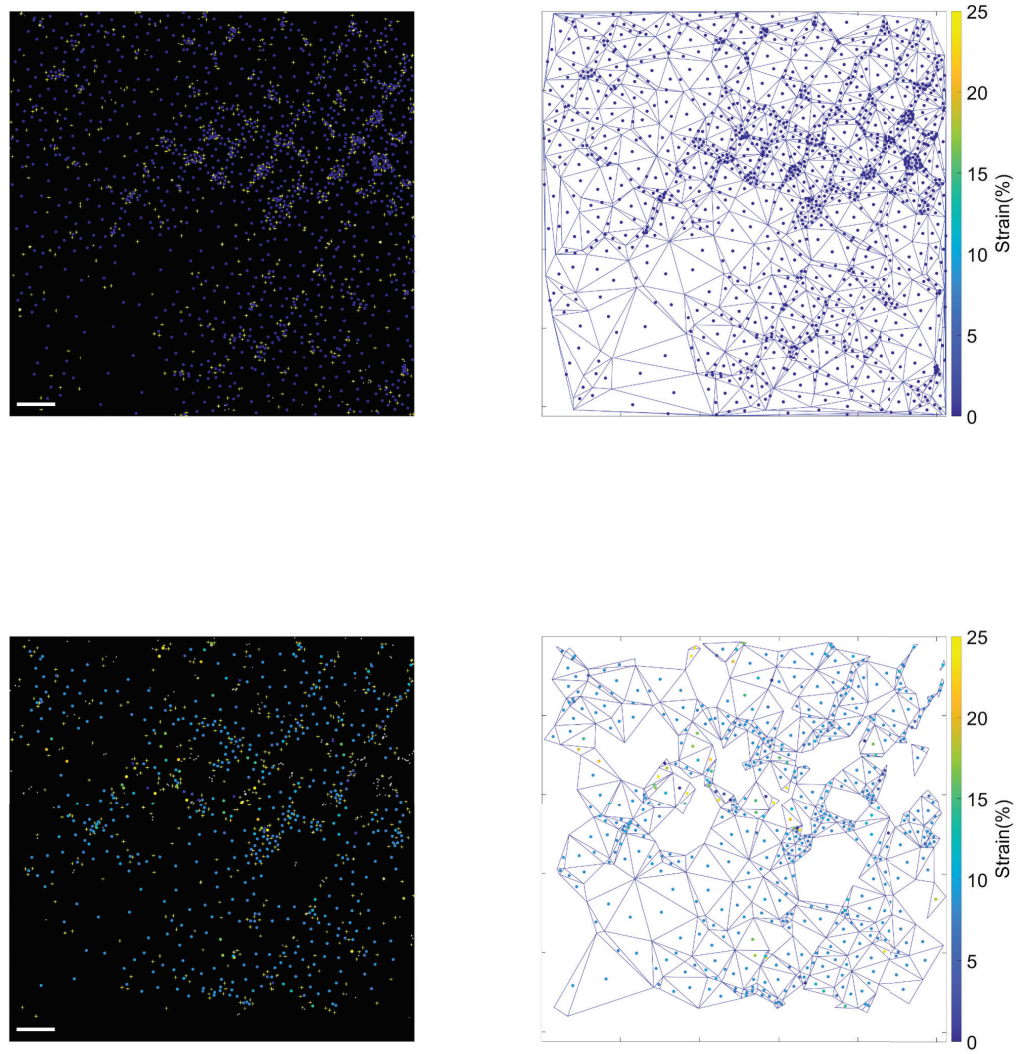


Figure 4.12: E_{xx} component of the strain field before (top panel, corresponding to frame 0) and during strain application (bottom panel, corresponding to frame 40). Each colored point represents the center of a triplet of tracked points. Line segments denote the members of each Delaunay triangulation associated with each colored point. Point color indicates E_{xx} strain magnitude calculated for that triplet of points. Colorbar indicates relationship between color and strain. Scalebar $\approx 125 \mu m$.

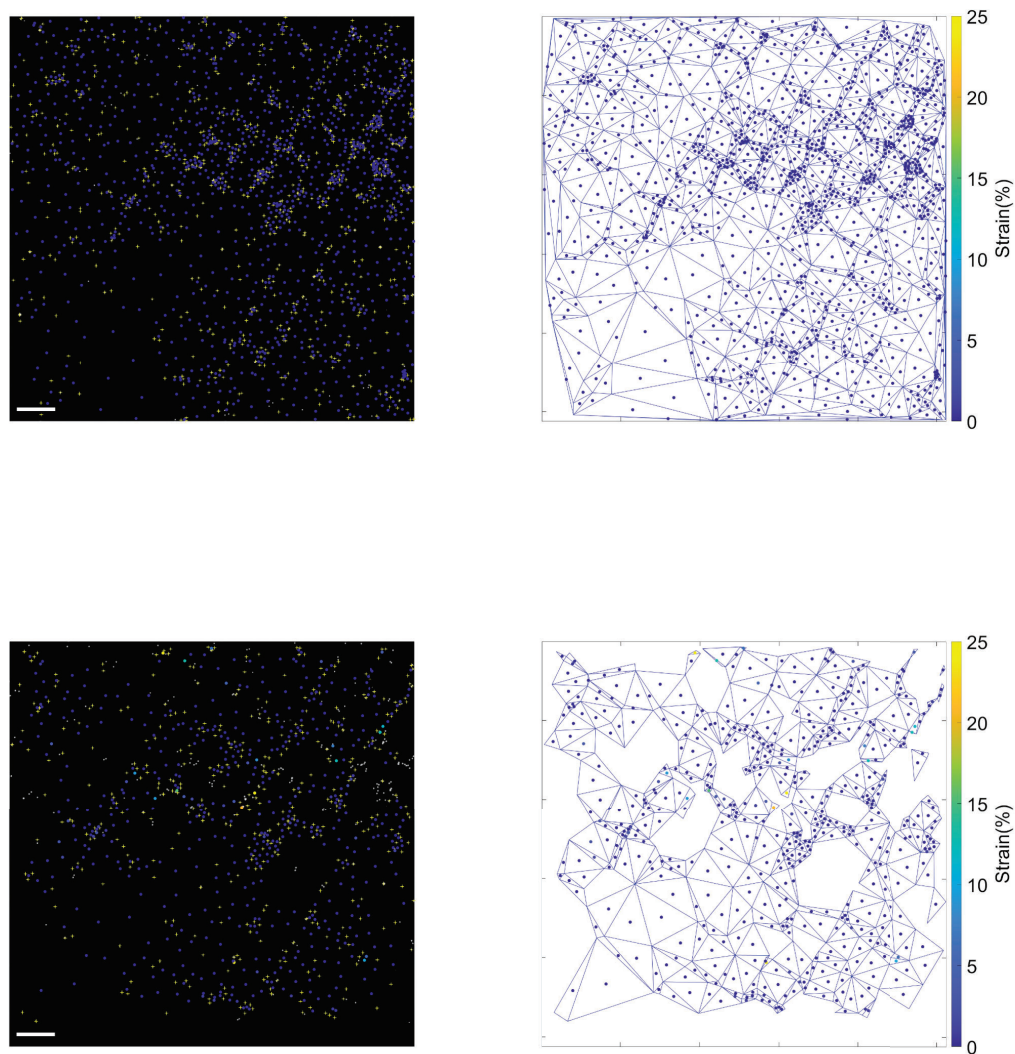


Figure 4.13: E_{xy} component of the strain field before (top panel, corresponding to frame 0) and during strain application (bottom panel, corresponding to frame 40). Each colored point represents the center of a triplet of tracked points. Line segments denote the members of each Delaunay triangulation associated with each colored point. Point color indicates E_{xy} strain magnitude calculated for that triplet of points. Colorbar indicates relationship between color and strain. Scalebar $\approx 125 \mu m$.

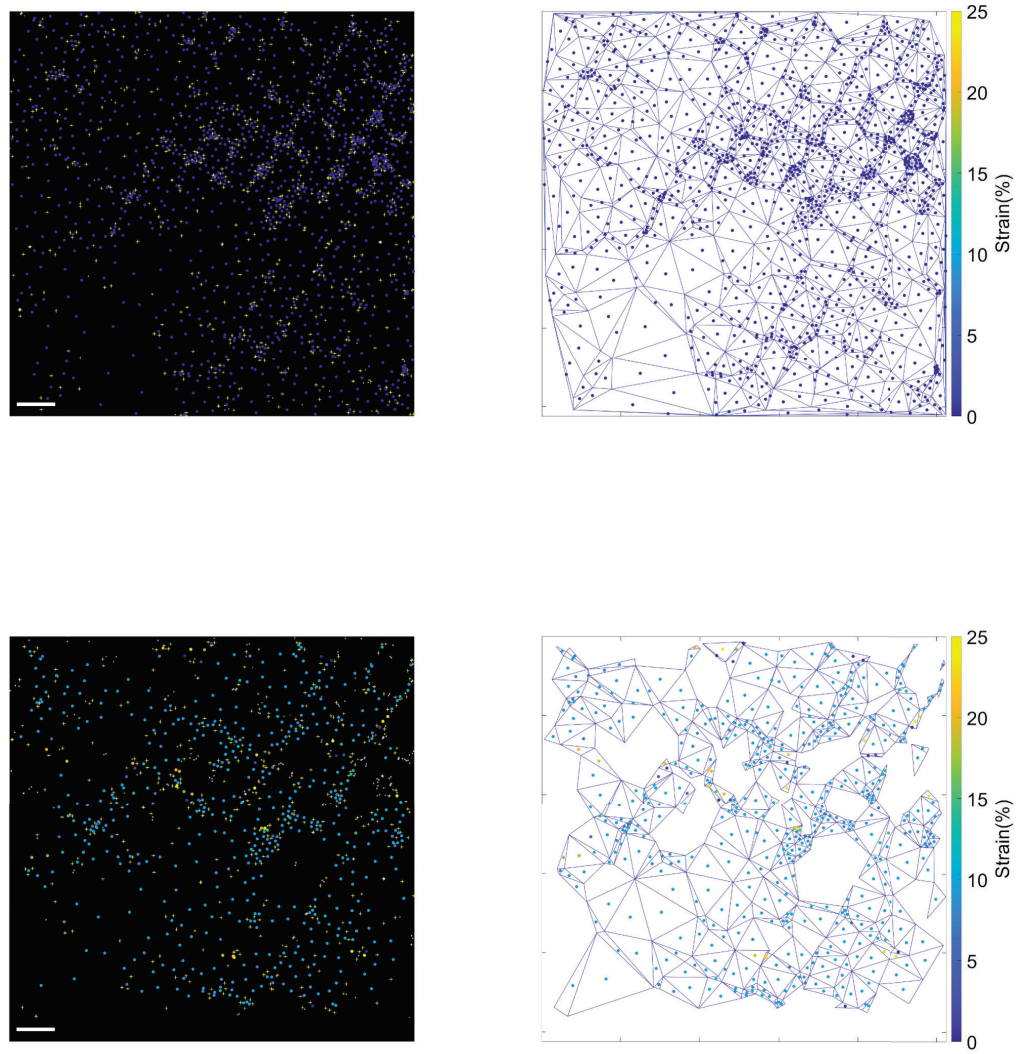


Figure 4.14: E_{yy} component of the strain field before (top panel, corresponding to frame 0) and during strain application (bottom panel, corresponding to frame 40). Each colored point represents the center of a triplet of tracked points. Line segments denote the members of each Delaunay triangulation associated with each colored point. Point color indicates E_{yy} strain magnitude calculated for that triplet of points. Colorbar indicates relationship between color and strain. Scalebar $\approx 125 \mu m$.

4.2.4 Discussion

The average normal strains measured by the strain tracker were not significantly different from each other in stretcher 1 and 2 and the shear strain measured was negligible for all cases, consistent with a state of equibiaxial strain, as is expected. The average strain measured in stretcher 3 plateaued after 3 turns rather than continuing to increase linearly as seen in stretchers 1 and 2. Upon manual inspection of the corresponding image data, this plateau did not appear to be an error in tracking, as the pattern did not appear to stretch in the corresponding frames. This plateau was thus likely due to a malfunction in the stretcher, most likely in the o-ring clamp for the membrane, which would allow the membrane to "slip" instead of continuing to deform. The results are also roughly consistent with the measurements made in [41] and [52], but are statistically different. This is likely due to differences between membranes for the same stretcher, or deviations in component dimensions due to inaccuracies in the manufacturing process, most likely in ring radius or thread pitch. Still, the possibility that the strain tracker overestimates the strain field cannot be dismissed without further investigation and validation. Nonetheless, the results are consistent with the analytical solution up to 20% equibiaxial strain, which is the maximum strain typically applied in SC cell stretching experiments. In other words, the strain tracker should provide accurate tracking in experimental strain ranges.

The results from the micropatterned substrate and fluorescent particle tracking experiments using the vacuum actuated stretcher indicate that the strain tracker is useful for cell scale data. This is expected, as the absolute size of the particles is irrelevant in the proposed tracking paradigm, which depends instead on the relative scale of the particles to the frame of the image and the resolution of the image data.

However, there was a significant amount of dropout in the tracked points. This is acceptable for overall strain field measurements as long as a reasonable proportion of the points remains, but is unacceptable for the study of local deformation, because loss of points mean loss of resolution and/or loss of measurements in certain areas. Dropout likely occurred because the displacement of fiducial markers between frames was too large relative to the average distance

between said fiducial markers, which makes registering the markers between frames difficult. One method of addressing this issue could be to increase the frame rate of video capture, so that the distance traveled by particles between frames is reduced to less than half the average distance between fiducial markers. These dropped points can prove especially problematic when they cause changes in triplet assignments, leading to erroneous strain calculations. These errors reduce the accuracy of the strain calculations and limit the usefulness of the algorithm.

The spacing between fiducial markers determines the spatial resolution of the calculated strain field. To calculate the strain distribution within cells, the grid resolution must ensure that there are multiple markers on each cell. To achieve this, the 2D concentration of fiducial markers within the field of view must be optimized to minimize the average distance between markers. This involves the selection of the optimal particle size and concentration in the incubation solution, as well as incubation time and particle surface treatment to ensure adhesion to the tracked surface to maximize coverage while still maintaining discrete resolvable points. As the resolution of the grid increases, the average distance between fiducial markers will decrease. This necessitates an increase in video frame rate as described previously, or a decrease in the speed with which strain is applied, to ensure that particles do not move too far between frames to track.

The proposed strain tracking system potentially allows for the investigation of how strain is distributed across the cell, providing the marker selection and application process can be optimized to minimize marker dropout. Tracking of strain within individual cells can be facilitated by culturing on a patterned surface that controls cell dispersion, which would also allow for control over cell morphology during culture and experimentation. This would allow for investigation into whether mechanical strain is transmitted from the substrate to the cells as expected.

The study of how strain is borne by different subcellular structures should also be possible with the strain tracking system detailed above, provided fiducial markers can be attached to said structures. For example, cell membranes could be coated with fluorescent particles and

mitochondria could be fluorescently labelled at separate wavelengths, then imaged in both channels in each frame.

By combining a strain tracking experiment using the strain tracker detailed above, with the fluorescent pore assay and pore detection system detailed in section 2, it should be possible to study the strain distribution in SC cells during pore formation. If mechanotransduction is important for triggering SC pore formation, which is likely given the findings in [8] and section 3, structures experiencing large strains within the cells may be involved in SC pore formation, and so the identification of these structures is of key importance in understanding SC pore formation, and how it is reduced in glaucoma.

4.3 A Method of Studying the Time Dependent Behavior of Transcellular Pores in Cultured Schlemm's Canal Inner Wall Cells

4.3.1 Introduction

Elevated intraocular pressure, the main risk factor for glaucoma, is caused by elevated resistance in the aqueous humour outflow pathway. The majority of this resistance is generated in the vicinity of the inner wall of SC [5]. SC inner wall cells experience large deformations in vivo. These deformations trigger the formation of pores [8], which act as permeability hotspots in an otherwise impermeable monolayer. These pores are thought to be the mechanism by which outflow resistance is controlled [22]. This makes SC pore formation a valuable research focus for understanding IOP regulation and treating glaucoma.

SC pore formation is still poorly understood, and it is currently unknown if SC pore formation is a dynamic process. It is possible that the reduction in pore density in glaucoma could be due to a decrease in the rate of pore formation, or alternatively an increase in their rate of closure. Further, one way to try to increase pore density in the inner wall could be to try to slow the process of pore closure. If this were to be the case, the study of pore dynamics, such as pore formation rate and closure rate, could be a useful method in the search for novel glaucoma therapeutic targets.

To investigate pore formation, a fluorescent assay was developed to label pores in SC cells [1]. The cells are stretched, which triggers pore formation, and exposed to fluorescent avidin that crosses the cell body at pore locations to create a fluorescent “hotspot” on the substrate that can be imaged by confocal microscopy. However, this and other established methods for studying SC pores [8] have no temporal resolution, making it impossible to investigate if and how SC pore density changes over time in a given population of SC cells.

We present below an improved method of studying SC pores based on the fluorescent assay

detailed in section 2.2 that allows for the visualization of pore dynamics, such as pore formation and pore closure, by introducing multiple staining time-points with non-overlapping fluorescent spectra, while retaining the benefits of said assay. We also present preliminary evidence that SC inner wall cells form pores when stretched in subconfluent culture, as well as preliminary evidence that SC pore density varies over time during static deformation in subconfluent SC cells, potentially indicating that pore formation is a temporally dynamic process and motivating further study of time dependent SC pore behaviour. Both observations are, to our knowledge, novel findings.

4.3.2 Methods

Experimental Design

To investigate whether pore locations change with time for a constant applied stretch, we extended the staining protocol developed in [1] to apply two different colored stains at different times after stretch. When imaged, each channel would give a snapshot of the pore locations in that staining interval, and comparing these two channels would show if differences existed.

To guide investigation of SC pore dynamics, we considered a simple rate based model for the dynamic control of pore density. The model describes the rate of change of pore density, N , over time t as a balance of pore formation rate, α , and pore closure rate, β . The model can thus be written as:

$$\frac{dN}{dt} = \alpha - \beta N \quad (4.17)$$

Where N , α and β may be functions of time, strain and/or other factors.

Assuming that pore formation and closure rates reach steady state equilibrium, this model would predict a steady state value of pore density according to:

$$N = \frac{\alpha}{\beta} \quad (4.18)$$

Experiments were conducted according to a protocol similar to that detailed in section 2.2.1. Equibiaxial cell stretchers manufactured according to the design established by Lee et al. in [41] (detailed in section 2.2.4) were used to apply mechanical strain to cultured SC cells. The PDMS membranes of these stretchers were covalently coated with biotinylated gelatin according to the protocol detailed in section 2.2.5 to allow for both SC cells and avidin based fluorescent stains to attach to the membrane surface. Gelatin was biotinylated from stock solution according to the protocol detailed in section 2.2.2. SC cells from one normal cell line (SC60) were cultured according to the protocol detailed in section 2.2.3. All experiments were performed by the author.

Schlemm's Canal Cell culture

SC cells were isolated from human donor eyes (SC60). SC cells were cultured in T75 flasks using low glucose DMEM containing 25 mM HEPES buffer (Gibco 12320, Life Technologies Co, USA), 10% fetal bovine serum (Hyclone SH30070.03, Thermo Scientific, USA) and 100 U/mL penicillin and 100 μ g/mL streptomycin (P4333, Sigma Aldrich, UK). Cells were incubated in 5% CO₂ at 37C. Cells were passaged prior to confluence using trypsin-EDTA (T4049 Sigma-Aldrich, UK).

Twist actuated cell stretchers were assembled and fitted with PDMS membranes coated with biotinylated gelatin. A cloning grid was placed on the surface of the membrane and sealed with silicone grease to form a 4x4 grid of experimental wells. Cells were seeded at 24,000 cells/cm² and left to incubate overnight in 5% CO₂ at 37C.

Staining Protocol

Prior to applying stretch, solutions of FITC-Neutravidin and TexasRed-Neutravidin were diluted to 25 μ g/mL in DPBS. These staining solutions were kept at 37C, along with DPBS

for washing and 4% ultrapure formaldehyde diluted in DPBS for fixation.

The staining protocol followed the following timeline (see Figure 4.15). At $t=0$ s 20% equibiaxial stretch was applied. At $t=90$ s (1min 30s), culture media was aspirated. At $t = 120$ s (2min 0s), cells were exposed to the FITC-Neutravidin staining solution. At $t = 270$ s (4mins 30s), the FITC-Neutravidin staining solution was aspirated and cells were washed twice with DPBS. At $t = 360$ s (6mins 0s), cells were exposed to TexasRed-Neutravidin staining solution. At $t = 510$ s (8mins 30s), the TexasRed-Neutravidin staining solution was aspirated and cells were washed twice with DPBS. At 600s (10mins 0s) the DPBS was aspirated and cells were fixed in ultrapure formaldehyde diluted to 4% in DPBS for 1 hour at room temperature. After fixation, specimens were cut out from stretcher membranes with a 6mm biopsy punch and mounted on microscope slides. Specimens were imaged on a Leica SP8 inverted confocal microscope (Facility for Imaging by Light Microscopy (FILM)) with a 20x dry objective, and images were inspected for pores manually using established criteria as detailed in 2.3.1.

Due to the subconfluent nature of the cell culture, paracellular pores could not be captured using this method. Thus, all pore counts and densities presented refer solely to transcellular pores.

Statistical Tests

Because pore counts tend to be Poisson distributed, traditional methods of comparing means such as the T test are inappropriate. To compare pore densities, which are normalized average pore counts, we used the E test developed by Krishnamoorthy and Thomson in [45], which allows for the comparison of Poisson distribution means. Where appropriate, a Bonferroni correction was applied.



Figure 4.15: Timeline of events during the two-timepoint staining protocol.

Stretch	Pore Count	Cell #	Cell Area Examined (mm^2)	λ	Pore Density (mm^{-2})
0%	14	39	0.147	0.358	95.1
20%	40	43	0.253	0.930	159

Table 4.1: Pore counts and densities obtained using the two-timepoint staining protocol on subconfluently cultured SC60 cells. λ is the mean of pore count and is calculated as n/k , where n is pore count and k is number of cells examined. Number of cells examined was defined as the number of dark regions surrounded by stained substrate examined. Regions that appeared to be clusters of cells based on visual inspection of region morphology were excluded.

4.3.3 Results

Schlemm's Canal Pore Density increases with Mechanical Strain in Subconfluent Culture

In SC60 cells, pore density significantly increased with stretch ($p < 0.05$, E test) from 0.359 pores per cell (95.1 pores/mm^2) at 0% strain to 0.930 pores per cell (160 pores/mm^2) (see Table 4.1). Pore densities observed are higher than previously reported by SEM studies (see Figure 4.16), but replicate the established trend of increasing pore density with strain.

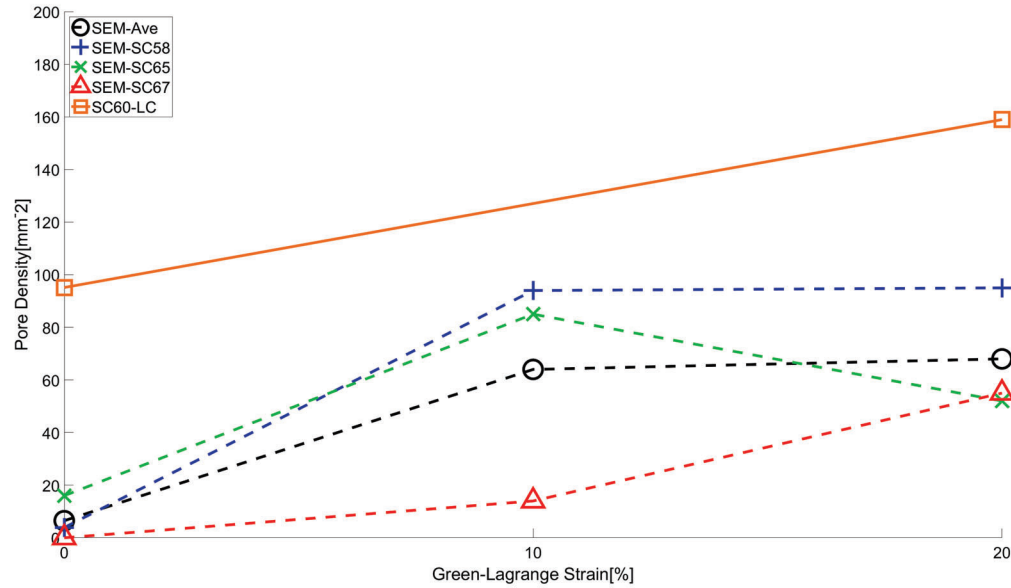


Figure 4.16: Transcellular pore density versus applied mechanical strain in low confluency SC cells (SC60-LC). Results from SEM studies reproduced from [8] for comparison (SEM traces).

Transcellular Schlemm's Canal Pore "behavior types"

There are observable differences in pore staining locations between the FITC and TexasRed stainings (see Figure 4.17 for examples), suggesting that pores exhibited time dependent behaviour, despite a constant applied mechanical strain. Three distinct transcellular pore behaviours were observed:

- "Full-Lifetime" - pores can colocalize, meaning they appear in both the FITC and TexasRed stainings. This most likely represents a pore that was open for both stainings, including the period between stain applications.
- "Early-Closure" - some pores were open during the FITC staining window but not during the subsequent TexasRed staining. This means the pore closed between stainings, despite constant applied stretch.
- "Late-Formation" - some locations showed no pore during the FITC staining window but did show a pore in the subsequent TexasRed staining. This means a pore formed after the first staining window, i.e. six minutes after stretch was applied.

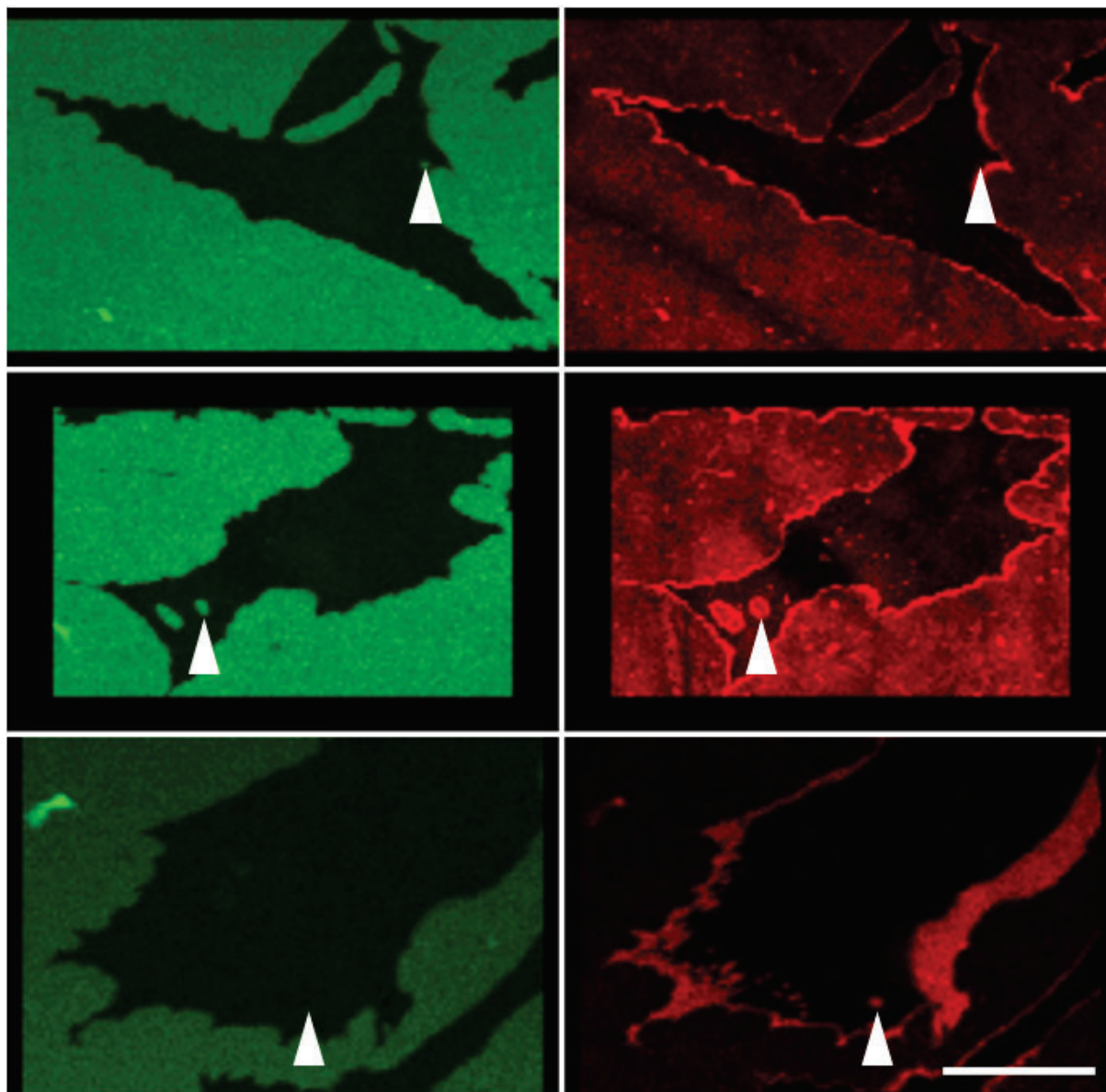


Figure 4.17: Examples of different pore "behaviors"; (Top panels) Early Closure, (Middle panels) Full-Lifetime and Late formation (Bottom Panels). Left panels show typical images from the first staining time-point. Right panels show the same cells at the second staining time-point. Pore locations are indicated by white arrowheads (arrowheads are matched in location between left and right panels to indicate pore absence at certain time-points). Scale bar = $30\mu m$.

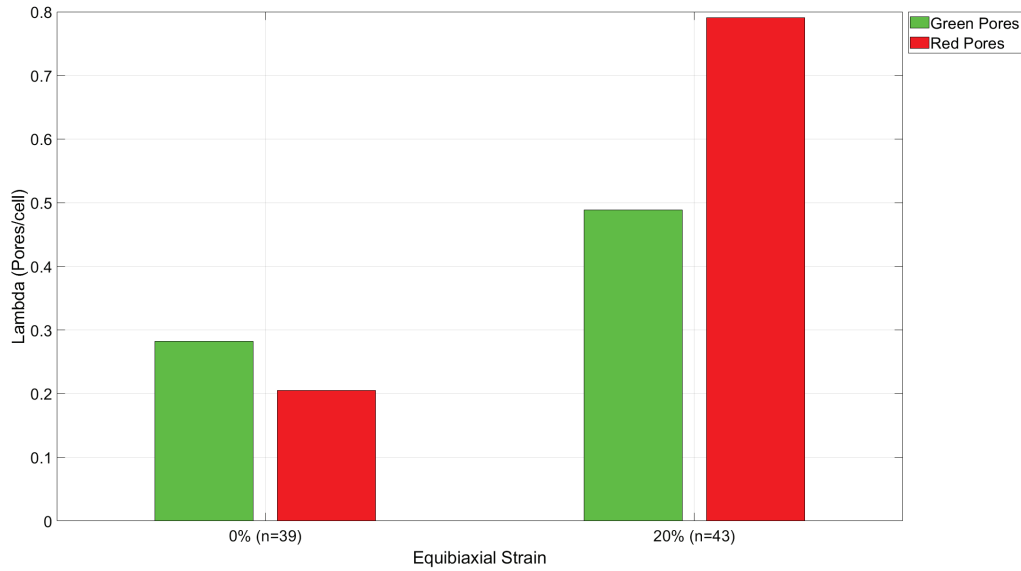


Figure 4.18: Total pore density at different experimental time points in different conditions. Green indicates pore density at time point 1, $t = 120-300$ s after stretch onset. Red indicates pore density at time point 2, $t = 360-540$ s after stretch onset.

Pore Density Variations Over Time

The assumed rate based model described above posits that pore density should trend towards a steady state value. To test this assumption, we investigated pore density values between time points for the same conditions.

Overall pore density was not statistically different between time points ($p > 0.05$, E test) (see Figure 4.18), which would seem to suggest that steady state was reached within the timeframe of the experiment. It is worth noting however, that the magnitude of differences in pore density is high, with pore density at time point 2 being 60% higher than in time point 1.

We next investigated when the increase in pore density noted above occurred. Pore density at time point 1 was not significantly different between stretched ($\lambda = 0.28$) and unstretched ($\lambda = 0.49$) states ($p > 0.05$, E test). Rather, the increase in pore density occurred only at time point 2, increasing from $\lambda = 0.21$ at 0% strain to $\lambda = 0.79$ at 20% strain.

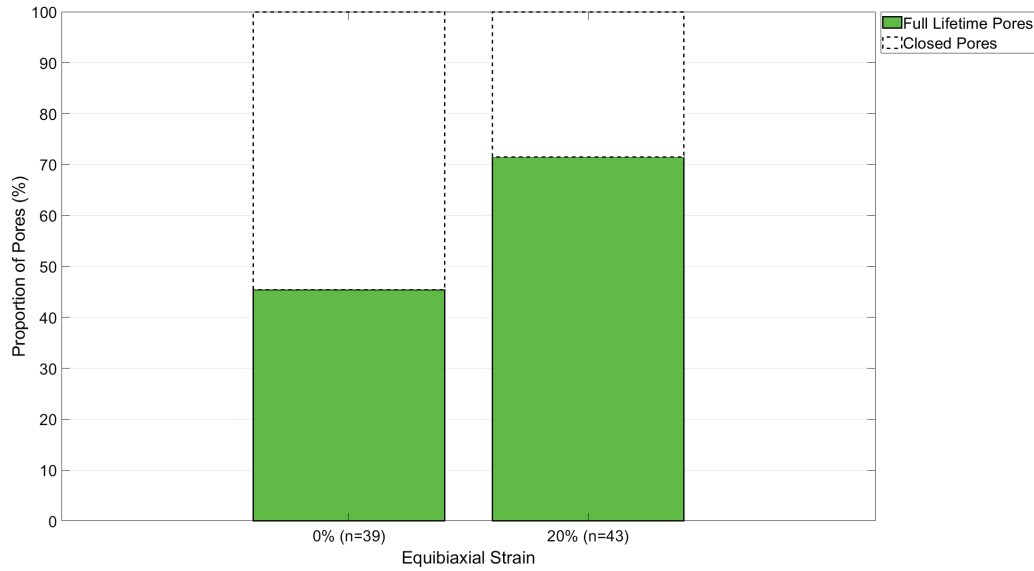


Figure 4.19: Ratio of pore closures to full lifetime pores at different stretch levels.

4.3.4 Effect of Strain on Pore Closure Rate

In the presented data some, but not all, pores close. This observation can be leveraged to investigate the effect of strain on pore closure rate β . Upon inspecting the proportion of observed pore closures (defined as the ratio of "closed" pores to "full-lifetime" pores), it was observed that fewer pore closures were seen at 20% strain (29% pores closed) than at 0% strain (55%) (see Figure 4.19). However, this difference was not statistically different ($p > 0.05$, E test).

Pore Type Distribution Varies with Mechanical Strain

The distribution of pore types changes with strain (see Table 4.2 and Figure 4.20).

Early-closure pore density showed no change with strain ($p = 0.8708 > 0.017$, E test with Bonferroni correction (n=3)). Full-lifetime pore density showed an increase with strain, but was only borderline significant ($p = 0.0379 > 0.017$, E test with Bonferroni correction (n=3)). Late-formation pore density significantly increased with strain ($p = 6.65 \times 10^{-4} < 0.017$, E test with Bonferroni correction (n=3)).

Stretch	Early Closure Pores	Full Lifetime Pores	Late Formation Pores
0%	6	5	3
20%	6	15	19

Table 4.2: Pore counts of different "pore types" obtained using the two-timepoint staining protocol on subconfluently cultured SC60 cells.

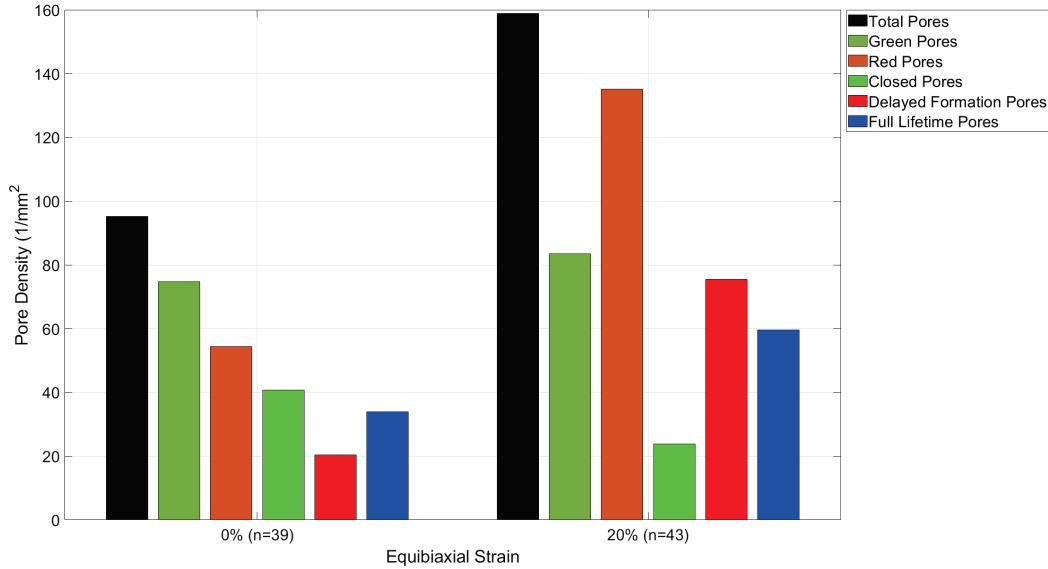


Figure 4.20: Graph of trends in pore density split by behavior type.

4.3.5 Discussion

SC cells cultured at low confluence retain their ability to form pores. Additionally, the SC pore density trends seen in previous studies are replicated in low confluence culture. This suggests that SC cells cultured at low confluence form pores in the same manner as other established methods. However, the low confluency pore densities reported here are much higher than in previously reported SEM data. This may be due to SEM underreporting due to the small cell area examined. This increase may also be due to increased cell spreading due to increased space per cell and lack of neighbouring cells in subconfluent culture, which may mean that the subconfluently cultured cells are thinner when stretched, facilitating the fusion of the basal and apical cell membranes. Encouragingly, it has been established that pore density values tend to vary by cell line. However, more cell lines must be subjected to low confluence pore assays to determine if the observed elevated pore density is inherent to low

confluence SC culture or an outlier. It should also be stressed that the findings discussed here are for a single cell line, and as such are highly preliminary. All findings should be confirmed by investigating additional cell lines.

These data indicate that transcellular SC pores are transient, even for a constant applied strain, which is a novel finding to the best of our knowledge. Pores form at different times regardless of what deformation is applied to the cells at the time of staining, and pore closure rate is an important factor in pore density at any given instant. Despite the dynamic nature of pore density, it seems that pore density does reach a steady state equilibrium, as suggested from the proposed model above. Pore formation rate does not significantly increase until $t = 6$ to 9 minutes after strain onset. This suggests that pore formation rate α may contain a time delay. Pore formation rate encapsulates the mechanotransduction process and the physical pore formation process, involving the fusion of basal and apical cell membranes. Processes such as membrane fusion are typically fast[53], but in order for membrane fusion to occur, the cytoplasmic contents of the cell must be moved out of the way. Perhaps, then, the processes of cytoskeletal rearrangement and/or flow of cytoplasm are the major influences on the observed time delay. For example, in blood platelets, F-actin reorganization occurs on a timescale of hundreds of seconds[54], which more closely matches the delay observed here. Pore closure rate β showed a large decrease with stretch, although this difference was not statistically significant. This suggests that pore closure rate may have a dependence on cell stretch, although further experiments must be conducted to see if a significant difference is detectable with more data.

Our findings indicate that late formations are significantly more likely and that more pores stay open for the duration between staining periods when the cells are under applied mechanical strain compared to when the cells are at rest. This intuitively explains why pore density increases with stretch, but it is noteworthy that pores are not directly linked to stretch in a simple "one-to-one" relationship, as their formation can be delayed from the onset of stretch. This delay could indicate that some form of signaling pathway may be involved in pore formation.

These findings highlight the need for increased temporal resolution in SC pore formation studies, to allow for quantification of delays between cell deformation and pore formation, as this may give insight into which cellular processes are involved in pore formation. More biological replicates on additional cell lines should be conducted to confirm if this behavior is replicated in SC cells from different donors. Glaucomatous cell lines should also be investigated, as any differences in the temporal dynamics of SC pore formation may provide new insight into how pore formation is impaired in glaucoma.

The ability to study pore formation with low confluence culture is valuable, since it means techniques such as substrate micropatterning can be used to control cell morphology during SC pore formation assays. Such a technique would involve culturing tens or hundreds of cells on a micropatterned substrate, such that each cell attaches to a spatially isolated island of culture surface and spreads to assume a culture area determined by the area of the patterned island. By controlling the number, location, size and shape of cultured cells, cell imaging can be more easily automated to produce reproducible and thus comparable results. This also has the benefit of reducing the amount of cells required to perform SC pore formation studies, which is highly desirable as SC cells are primary donor cells and thus very rare and valuable. Because fewer cells are needed, experiments can be performed at lower passage numbers, which is always desirable in cell culture as cell phenotype tends to degrade with passage number.

Low confluence culture also largely removes the difficulty associated with transcellular pore identification in the image data. Because low confluency means cells have large areas of bare culture surface surrounding them, cells cultured at low confluence do not share borders with neighbouring cells. This means that the border of each cell is clearly identifiable, and thus the challenge present in confluent data of distinguishing pores from leaky junctional staining is avoided. However, it should be noted that paracellular pore detection is impossible using subconfluent culture, since cells no longer neighbour each other, and so cannot form junctions.

The proposed adaptation to the fluorescent pore assay currently has several shortcomings, some of which can be overcome with optimization. It should be noted that paracellular pore

formation cannot be studied using low confluence SC cell culture, and that the majority of SC pores tend to be paracellular. Other techniques must be developed alongside the methods described above to gain a full understanding of SC pore formation. The membrane coating procedure used also proved problematic, with artefacts due to cracked substrate coating greatly affecting image quality. This can be addressed by altering the coating protocol to avoid cracking.

Chapter 5

Conclusions and Recommendations for Future Work

5.1 Summary of Thesis Achievements

This thesis has provided tools and methods to enhance study of SC pore formation. In the past SC pores have primarily been studied using SEM, but this method is time consuming and low-throughput. A promising alternative to SEM has recently come forward in the form of a fluorescent pore assay. This fluorescent assay is cheap, fast and scaleable to a high-throughput workflow. However, due to the inability of SC cells to form tight junctions in culture, image data from this assay is difficult to manually analyze, particularly for confluent SC cells, creating a bottleneck. The machine learning system developed in Chapter 2 for the task of identifying pores in the image data obtained from the aforementioned fluorescent pore assay reduces this bottleneck. The machine learning model itself demonstrates high discriminative capacity, given the high F1 score of the final model, but is held back by low precision due to the large class imbalance inherent in the dataset. When combined with the masking protocol, criteria for pore classification, and manual validation application detailed in Chapter 2, this low precision is overcome, and the image data can be analyzed in a fraction of the time compared to purely manual analysis.

The methods described in Chapter 2 were applied to the repository of image data produced by STB using the fluorescent pore assay in Chapter 3 to investigate SC pore density in six cell lines (three normal, three glaucomatous). Our findings demonstrate that transcellular pore density increases with stretch across three normal cell lines, concurring with previous findings. We also find that transcellular pore density is reduced in glaucomatous, relative to normal, SC cells exposed to the same substrate stretch. It is interesting to note that while previous studies have detected reduced pore density for the same pressure drop, we are able to detect decreased glaucomatous pore density for the same applied deformation. This means that, while previous trends may have been explainable due to the increased subcortical stiffness of glaucomatous SC cells, the findings presented here suggest that SC cell stiffness alone cannot explain reduced pore forming ability in glaucomatous SC cells.

In our analysis in Chapter 3, we detected a significant increase in pore area with stretch. This contrasts with the findings in a previous study [8], which saw no change. This previous study used SEM to identify and measure pores, and thus investigated a far smaller total cell area and as a result identified fewer pores. This, combined with the possibility that a large proportion of pores observed in SEM may be fixation artefacts, suggests that this trend may have been missed due to the limitations of SEM as a tool for pore identification. We also detected a significant decrease in glaucomatous pore area. These findings have implications for our understanding of how outflow resistance is modulated. Because AH likely drains via SC pores, their area directly effects their hydraulic resistance. If pore area increases with stretch, then the outflow resistance contribution from SC pores generated by a given section of the SC inner wall is inversely proportional to the deformation experienced by that section. Because SC pore formation itself is induced by cellular deformation, there is a compounding effect where stretched cells produce, on average, more pores and larger pores, combining to produce a higher facility than either effect would suggest alone. Similarly, if glaucomatous cells form smaller pores on average, then the SC inner wall in glaucoma would be more resistive to outflow not just due to their increased stiffness, reducing the amount of deformation and thus the likelihood of pore formation, or their reduced ability to form pores, but also due to the pores formed being smaller than those of normal cells; These effects would

again compound to increase outflow resistance.

The analysis presented in Chapter 3 then examined the shape of transcellular pores by comparing pore eccentricity and solidity, finding some minute differences between experimental conditions which are unlikely to be physiologically relevant. It is possible these differences may have been due to pores being stained in the process of closing, but it is impossible to confirm as the low resolution of the image data and the lateral diffusion of the stain under the pore make it difficult to discern whether pores with lower solidity were in the process of closing or partially obscured in some way. Despite the lack of significance, the ability to obtain these more complex properties of pores from the data with negligible additional processing time serves to highlight the power of the processing pipeline developed.

All published studies of pore formation have focused on imaging fixed pores at single time points. Yet, we know that the inner wall is highly dynamic, changing in response to pressure [55] and over time at a fixed pressure [56]. Hence, we investigated the time-dependent nature of pore formation. The method of studying pores detailed in Section 4.3 allows for investigation of SC pores over time. The preliminary data collected using this method already provides new insights, showing that SC transcellular pores are dynamic structures. We also present evidence that there may be a time delay to pore formation, which previous experiments may have missed. Additionally, our findings indicate that the rate of pore closure may be stretch dependent. However, these results are from experiments on a single cell line and thus must be taken as preliminary. Despite this, the study demonstrates the need for time-lapse studies of SC pores, and the insights that can be uncovered with the presented modifications to the fluorescent pore assay.

The structures associated with the transduction of deformation into pore formation are unknown, and the process itself is still poorly understood. To investigate this aspect of pore formation, a method of quantifying the mechanical strain within the cell is required. The algorithm presented in Section 4.2 is capable of performing this role, given additional work to optimize both the algorithm and the experimental protocol for applying fiducial markers to cellular structures of interest. Tracking strain in the cell membrane should be relatively simple

by adsorbing fluorescent nanoparticles onto cultured SC cells. Tracking strain in internal structures, such as the cytoskeleton, may prove more challenging, but as long as a method of producing trackable points labelled to those structures can be found, the strain field across the cell can be estimated. The presented algorithm offers an exciting method of investigating SC pore formation from a previously unexplored angle, and can be expanded to the study of other scenarios with little modification, since it functions at any scale, at any time resolution; It is limited only by the size, contrast and density of the field of fiducial markers.

5.2 Future Work

The work presented in this thesis enhances our ability to investigate SC pore formation, and provides new insights into SC pore formation and the effects glaucoma has on SC outflow resistance modulation. However, it also raises several questions that can be addressed using the techniques presented. Here we present some immediate next steps that should be pursued to further deepen our understanding of SC pore formation. These steps are presented in order of priority, in terms of feasibility and order of dependency.

Establish a Method of Controlling Schlemm's Canal Cell Location during Culture

Our understanding of pore formation is still rudimentary, and so we seek methods of better understanding the pore formation process. Subconfluent culture is a promising step towards that goal, because it requires fewer cells and makes cell location and indexing a simpler process, enabling the investigation of individual cells. However, if cell thinning and the fusion of apical and basal membranes are needed for pore formation to occur, cell thickness, and therefore morphology, need to be controlled when investigating pore formation. Subconfluent culture itself does control for cell morphology, and the random arrangement of cells with no landmarks for orientation means that cell indexing is still a non-trivial problem.

Cell culture micropatterning via soft lithography may provide a solution to both of these issues. Soft lithography is the application of micro-scale manufacture to microbiology and concerns the creation of a pattern on a substrate using a "master" reference[57]. Typically, a

”master” will be produced by using photolithography to crosslink a CAD-designed pattern of photoresist onto a silicon wafer. This master will then be used to produce a copy of the pattern on the substrate, typically by producing a stamp or mould from the master, depending on the application.

By using soft lithography techniques to pattern the culture substrate with islands of culture surface, surrounded by regions coated with cell-repellent polyethelene glycols (PEGs), cells can be encouraged to grow in specific regions of the substrate. By organizing these islands into a grid, and including landmarks in the grid as reference points, the islands on the substrate can be easily indexed. By controlling the size and shape of the islands, subconfluent culture can be enforced, such that individual cells can be indexed, and cell morphology can also be controlled.

Micropatterning presents great potential advantages to the field of mechanobiology, but there are several challenges that are unique to SC pore research that must be overcome. For SC cells to be stretched, they must be cultured on a stretchable, yet biocompatible substrate. To be studied via microscopy, the substrate must be transparent. Each experiment requires a new substrate, and so the substrate must be inexpensive to purchase, or the materials and techniques required to manufacture it must be affordable and easily performable in a laboratory with minimal extra equipment, respectively. To ensure strain is transferred from substrate to cell, any coating applied to the substrate should be covalently bound to the substrate surface. These constraints mean that the choice of substrate is very limited, with PDMS currently used for all SC stretch experiments. Stamps produced by soft lithography are typically made of PDMS, and are typically used on stiff substrates, such as glass.

Ensuring good transfer of pattern from stamp to substrate requires good contact between the two objects, and is made more complicated when the substrate is soft. Moulding the PDMS substrate in the desired pattern may be possible, but would mean that the mechanical properties of the stretched substrate would vary spatially, making application of a desired strain to cells more challenging.

The most likely method of achieving a micropatterned substrate for this application, then,

may be direct UV-crosslinking to the substrate. This would theoretically involve a UV-resistant mask, but as a "master" itself rather than a tool for the "master's" creation as is typically seen in soft lithography. Once designed and manufactured, this mask would be applied to a PMDS membrane coated with a UV-activated crosslinker. Applying UV light would bind the crosslinker to the substrate surface in the desired pattern. Subsequently, after washing the remaining unbonded crosslinker, the biotinylated gelatin required for pore staining experiments could then be crosslinked as normal, since only the pattern of areas desired would be able to react with the gelatin. PEGs could be adsorbed onto the unpatterned remaining surface to produce the desired grid of cell culture enclosures discussed above.

Investigate whether Schlemm's Canal Transcellular Pores Detected in The Fluorescent Pore Assay and SEM Colocalize

When using the fluorescent pore assay for pore identification, it is currently assumed that the blobs of fluorescence that meet the criteria established in section 2.3.1 colocalize with a transcellular pore, thus allowing for the measurement of pore density to be conducted using the fluorescent assay instead of the more time consuming SEM. This assumption has not yet been formally tested by examining the cells to confirm this colocalization. The best way to examine the cells themselves for pores remains SEM. Thus, a study should be conducted to test the aforementioned assumption and thus confirm that the fluorescent pore assay is a useful method of labelling SC transcellular pores. In such a study, cells from several cell lines should be subjected to the fluorescent pore assay, then fixed. Cells would then be imaged for fluorescence and via SEM. If a high degree of colocalization is observed, the assumption can be assumed to hold.

To perform such a study, the same cells must be examined with both methodologies. For this to be feasible, there must be a structured and reliable method of finding a particular cell; the cells must be organized in their arrangement and indexable. Micropatterning of the culture substrate is likely a feasible method of achieving this.

Both confluent and subconfluent culture should ideally be tested in such a study. However, it is unclear how a confluent culture is compatible with a micropatterned substrate with single

cell islands. An intermediate approach to micropatterning could be considered, where strips of culture area are patterned, rather than individual islands. This would produce a culture surface of several small sections large enough to contain groups of cells at confluence. These sections would then be indexed, rather than each cell itself. Because the strip boundaries are known, if the entire strip is imaged *en-face* from the same angle in both modalities, it should be possible to register the images from the two methods together to investigate colocalization. The dimensions of these micropatterned strips can be set such that each edge has a different length, preventing errors in registration due to rotations of the sample between imaging modalities. However, because transcellular pores have been detected using the fluorescent assay in subconfluent culture, and because subconfluent SC culture is preferred for pore studies due to the factors discussed previously, pore colocalization in subconfluent culture should be prioritized if possible.

Continue the Study of Schlemm's Canal Pore Dynamics

The findings presented in section 4.3 are exciting, but only come from a single cell line, and so must be treated as preliminary. The two-time point staining experiments should be repeated on additional cell lines to investigate whether the preliminary results presented are recreated in other cell lines. Glaucomatous cell lines should also be tested examined with the same methodology to investigate whether SC transcellular pore dynamics are altered in glaucoma. There may be more to the picture of how glaucoma alters outflow resistance buried in the time dynamics of SC pore formation, and so investigating the similarities and differences between normal and glaucomatous SC pore dynamics may offer new insights into the mechanism by which glaucoma is linked to increased outflow resistance.

Develop a Method of Studying Schlemm's Canal Pores in Three Dimensions

Cells are three-dimensional structures. When an object is subjected to a two-dimensional deformation such as an equibiaxial strain, the object's size in the third dimension will decrease if the volume of the object is conserved. SC cells are studied under such a deformation, yet little is known about how this third dimension, here the cell height, is affected during stretching. It has been postulated that SC pore formation, like pore formation

in other endothelia, requires the local thinning of the cell such that the cell's apical and basal membranes meet [27]. If SC cell deformation can be quantified in three dimensions, this hypothesis can be tested by examining the thinness of the cell in regions where pores form. Answering this question would improve our understanding of the requirements for and mechanisms of pore formation, further informing the list of possible targets for outflow resistance modulating drugs.

To study this, a method of measuring cell thickness changes is required which is compatible with methods of detecting SC pores. One possibility is to use confocal microscopy and fluorescent beads to calculate height. If fluorescent beads are attached to the substrate surface and to the apical surface of the cultured cells, the location of beads on the substrate can be used as a reference point and the height of a cell can be taken as the difference between the z coordinate of the reference bead and the bead on the cell. If cell footprint is controlled using micropatterning as discussed above, the cell's volume can then be inferred using the footprint area and measured height of the cell.

Another possible solution is to use the Airy disk patterns of fiducial markers placed on the cell to estimate the height of each marker while tracking its position in the field of view, which would allow us to infer the third dimension from a two-dimensional measurement and thus compute cell strain in three dimensions without necessitating the use of more complex imaging modalities such as 3D microscopy, which may not be compatible with methods of SC pore detection.

Develop a method of observing Schlemm's Canal Pore Dynamics in Real-Time

We have presented preliminary evidence demonstrating that SC pores exhibit time dependent behavior. SC pore formation and closure rates may be important in understanding how pore density is reduced in glaucoma. The modified staining protocol presented in chapter 4.3 allows us to investigate how pores behave over an arbitrary time period. However, two staining time-points provides very little temporal resolution for measurements, providing only a limited picture of the phenomena we wish to study. Adding more stains for additional time-points quickly becomes impractical, as each stain must be selected so as to avoid

overlapping the other stains in excitation and emission spectra. There is also the issue of finite biotin available for the stains to bind to, meaning that stain concentrations must be optimized to avoid saturation and to allow for consistent staining at later time-points. The process of staining takes tens of seconds, putting a floor on the time between staining time-points which may be too long to detect the relationships being investigated. This means that a higher time-resolution method of studying SC pore dynamics is needed.

In order to move towards a higher time-resolution method of studying pore dynamics, a fundamental shift in how pores are detected must be made. The answer may lie in the use of quenchable fluorescent probes or a similar schema where the state of a light emitter can be flipped from on to off or vice versa through contact with a secondary molecule. Such an experimental paradigm could feature a culture substrate covalently labelled with an even layer of light emitting probes in the "off" state and a culture medium containing a molecule which switches the state of the probes to "on" when it makes contact, similar to the biotin-avidin based fluorescent pore assay. Ideally, this signal would remain "on" for the duration that the pore stays open, allowing for fully real-time recording of SC pores.

One possible method of working towards this may be through the use of a quenched coumarin-based dye and a thiolated substrate (Dr. Sylvain Ladame, personal communication). In such case, the gelatin coating for the stretcher membrane would be thiolated in a similar fashion to that used for biotinylation in Section 2.2.2. The quenched coumarin-based dye would be dissolved in the culture medium. As regions of high permeability in the cell layer formed, the coumarin-based dye in solution would interact with the thiol, unquenching it and producing a fluorescent signal at that location. Another method of producing a signal as pore formation occurs may be through the use of Förster resonance energy transfer (FRET), where the recorded spectrum is bandpass filtered to capture the signal due to the FRET effect.

Investigate the Link between Applied Mechanical Strain and Pore Formation in Schlemm's Canal Inner Wall Cells

It has been established that SC pore formation is triggered by mechanical strain, but the relationship between the strain in the SC cell and pore formation is still poorly understood.

By examining the distribution of mechanical strain within the cell, or even within subcellular structures such as the cell membrane or cytoskeleton, and tracking pore formation simultaneously, it should be possible to begin linking the strain within the cell to pore formation, which may provide new insights into the specifics of the transduction mechanism that triggers pore formation, which may lead to new targets for glaucoma therapeutics.

This can be done using the strain tracker discussed in Section 4.2 together with the fluorescent pore assay described in Chapter 2. The adapted two time-point assay described in Section 4.3 can be used instead to investigate if and how changes in pore density over time correspond to changes in strain distribution over time. Because pore formation is induced in subconfluent culture, micropatterning of the culture substrate can be leveraged to control the shape of the cells. This would allow for the study of pore formation and strain on a cell-by-cell basis rather than across a monolayer.

Chapter A1

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Publication Title	Investigative ophthalmology & visual science	Country Rightsholder	United States of America Association for Research in Vision & Ophthalmology (ARVO)
Author/Editor	Association for Research in Vision and Ophthalmology.	Publication Type	e-Journal
Date	01/01/1977	URL	http://www.iovs.org/
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Title	Computer vision tools to automate the study of Schlemm's Canal inner wall cell biomechanics and pore formation	Institution name	Imperial College London
Instructor name	Prof. Darryl Overby	Expected presentation date	2022-10-01

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