Replicating Landmine Blast Loading in Cellular *in Vitro* Models

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1 Ultrasound and bulk density rig measurement

1.1 Ultrasound and bulk density rig characterization

The measurement rig consists of the assembly of a ring made of aluminium, a glass coverslip, a time delay line made of polycarbonate, and a flange to secure the delay line on the ring (**figure 1a**). A total of 12 rigs of 4 different nominal thicknesses (3, 6, 10 and 14 mm) were machined. The nominal inner and outer diameter of the rings are 30 mm and 52 mm, respectively. The actual thicknesses and diameters of each ring were accurately measured by using a calibrated digital micrometer (Mitutoyo, model 293-821-10, 0-25 mm, 0.001 m resolution) and a caliper (RS Digital Caliper, 877-1775, 200 mm, 0.01 mm resolution), respectively. Three sets of measurements were taken every 60˚ angle along the edges of each ring. The final dimensions of the rings are summarised in **table 1**. The error on the thickness and the diameter corresponds to the standard deviation of the measurement average (n = 18). The error on the diameter was determined by error propagation analysis of the thickness and diameter errors.

Table 1 Ultrasound and bulk density rig characterization. Average data (repeat = 18) expressed as mean ± SD. (‡) mean ± Δu, with Δu determined by error propagation analysis

Rig	Thickness, (mm)	Diameter, (mm)	Volume, (cm^3) ‡
#1	2.890 ± 0.003	29.782 ± 0.008	2.013 ± 0.003
#2	2.909 ± 0.003	29.780 ± 0.011	2.026 ± 0.004
#3	2.911 ± 0.003	29.780 ± 0.015	2.027 ± 0.006
#4	5.881 ± 0.002	29.783 ± 0.008	4.097 ± 0.004
#5	5.881 ± 0.002	29.767 ± 0.010	4.093 ± 0.004
#6	5.886 ± 0.002	29.868 ± 0.008	4.110 ± 0.004
#7	9.484 ± 0.007	29.858 ± 0.008	6.641 ± 0.008
#8	9.665 ± 0.004	29.783 ± 0.008	6.733 ± 0.007
#9	9.652 ± 0.009	29.777 ± 0.008	6.721 ± 0.010
#10	13.776 ± 0.001	29.843 ± 0.008	9.636 ± 0.006
#11	13.795 ± 0.003	29.778 ± 0.004	9.607 ± 0.004
#12	13.793 ± 0.003	29.773 ± 0.005	9.603 ± 0.005

1.2 Ultrasound hardware and testing

The ultrasound hardware consisted of a 1/2 inch diameter contact transducer, nominally 5 MHz (Olympus-NDT, model A109S-RM), an ultrasonic pulser-receiver (Panametrics-NDT, model 5058PR) and a digital oscilloscope (Tektronix, model DPO 2024, Portland, Oregon, USA). The rigs were designed to perform the measurements of speed of sound in the PDMS and hydrogel samples in pulse-echo mode. In pulse-echo mode, the contact transducer performs both the sending of the pulses and the receiving of the echos. On the pulser-receiver, the pulse repeat frequency, energy and damping resistance were set to 2 kHz, 891μJ and 500 Ω, respectively. The gain was set to 40 dB and a high-frequency hardware filtering was used at 0.1 MHz. All coaxial cabling was terminated with 50 Ω impedance. The oscilloscope was triggered by the pulser-receiver external trigger signal. For the ultrasonic evaluation, the measurement, containing the hydrogel, was coaxially placed on the top of the ultrasound transducer via the window hole in the flange. Depending on the ring thickness, the height of the delay line was carefully preset to introduce a time delay between the generation of the sound wave and the arrival of any echo. Light fine oil lubricant was used as couplant to carry the wave energy across boundary between the transducer and the delay line. Ultrasound tests were performed at room temperature (23 ± 2 ˚C).

1.3 Ultrasound and bulk density rig characterization

For a sound wave that propagates only in one direction, the acoustic impedance Z is found to be:

$$Z = \rho c_L \tag{1}$$

where, ρ and c_L are the bulk density and the longitudinal speed of sound in the material, respectively. In the special case of a wave transmitting perpendicularly to the boundary plane, the reflection ratio is given by:

$$R = \left(\frac{Z_1 - Z_2}{Z_1 + Z_2}\right)^2 \quad (2)$$

where Z_1 is the acoustic impedance of the medium of the incident wave, Z_2 that of the medium behind the boundary plane. The mass of the sample was determined by weighing the rig before and after casting the materials. We measured the transit time Δt for the pulse to perform a single round trip Δx across the sample (**figure 1b).** The longitudinal wave speed c_L was, then, determined as the slope of the least squares linear regression fit performed on the data points in the 'Δt versus Δx*'* parameter space (**figure 1c,d,g,h**). The bulk density ρ was computed in a similar procedure in the '*volume* versus *sample mass*' parameter space (**figure 1e,f,i,j**). The uncertainty on the sound speed and bulk density results corresponds to the standard error of the estimated slope of the linear fitting.

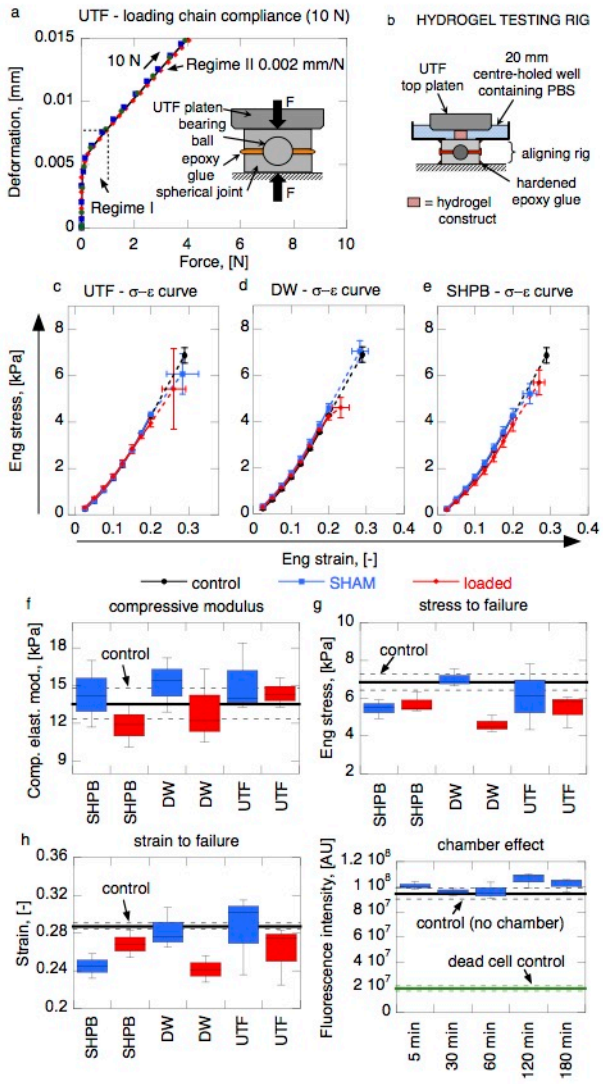


Figure 1. Longitudinal sound speed and bulk density measurements. (a) Testing rig and sample preparation protocol. (b) Representative time-history of ultrasound traces illustrating the echos from the top and bottom surfaces of the sample. DL = delay line. S = delay line + sample. (c-f) Longitudinal speed of sound and bulk density measurements in 1% (wt/v) agarose hydrogel. (g-j) Longitudinal speed of sound and bulk density measurements in polydimethylsiloxane (PDMS).

2 Calibration of the confinement sleeve and the loading cap

The calibration relationship between the output voltage and the strain was empirically determined by subjecting the sleeve to a radial loading, and then solving the circumferential strain distribution using the analytical solution of circular ring subjected to two equal forces acting along the diameter (**figure 2a)**. The calibration relationship between the voltage output and the applied axial force was determined by subjecting the loading cap to a series of compression-release cycles on a UTF (Instron, model 5866, Buckinghamshire, UK) (**figure 2b**).

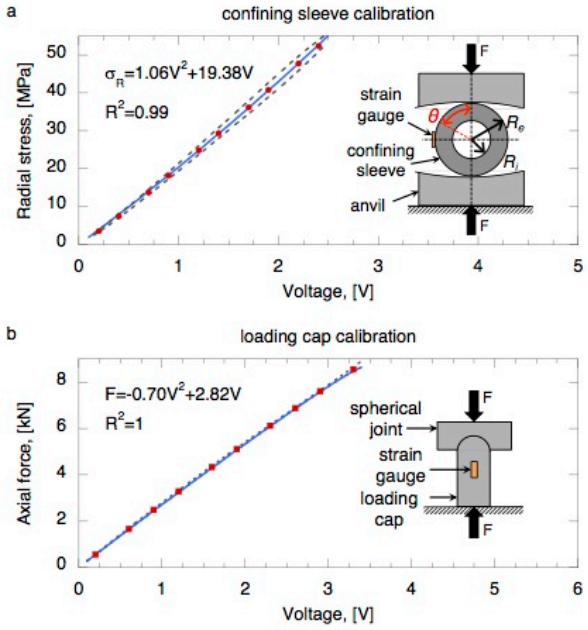


Figure 2 Confining sleeve and loading cap calibration. (a) Calibration of the confining sleeve. (b) Calibration of the loading cap.

3 *In vitro* platform calibration relationships

The calibration formulations between the characteristics of the loadings and the initial loading conditions were obtained from least-squares regression analysis on the data points (**table 2**).

Table 2 Summary of the impact conditions.

Platform	Impact conditions	Parameter, y	Unit	Model, $y = f(x)$	R (p value), R^2	Validity, x
SHPB	160 mm	Pressure	MPa	$y = 3.2861\ x - 10.035$	- , 0.99	$5 < x < 30$ m.s^{-1}
		Pulse duration	µs	$y = -0.1711\ x + 65.124$	-0.42 (0.12), 0.41	$5 < x < 30$ m.s^{-1}
	320 mm	Pressure	MPa	$y = 3.2025\ x - 4.1203$	- , 0.99	$5 < x < 30$ m.s^{-1}
		Pulse duration	µs	$y = 0.1323\ x + 104.83$	0.11 (0.70), 0.12	$5 < x < 30$ m.s^{-1}
	450 mm	Pressure	MPa	$y = 3.2236\ x$	- , 0.99	$5 < x < 25.3$ m.s^{-1}
		Pulse duration	µs	$y = 0.1554\ x + 155.43$	0.63 (0.15), 0.61	$5 < x < 25.3$ m.s^{-1}
	560 mm	Pressure	MPa	$y = 3.1886\ x$	- , 0.99	$5 < x < 21.5$ m.s^{-1}
		Pulse duration	µs	$y = 0.2956\ x + 195.68$	0.36 (0.19), 0.61	$5 < x < 21.5$ m.s^{-1}
DW	360 g	Pressure	MPa	$y = 0.5639\ x^{0.6841}$	- 0.001 (0.99), 0.99	$100 < x < 1{,}800$ mm
		Pulse duration	ms	$y = -6\ 10^{-7}\ x + 0.53$	- , 0.0005	$100 < x < 1{,}800$ mm
	560 g	Pressure	MPa	$y = 1.0018\ x^{0.636}$	- , 0.99	$100 < x < 1{,}700$ mm
		Pulse duration	ms	$y = -1.002\ 10^{-5}\ x + 0.77$	-0.09 (0.71), 0.13	$100 < x < 1{,}700$ mm
	1060 g	Pressure	MPa	$y = 1.4525\ x^{0.6111}$	- , 0.99	$100 < x < 1{,}250$ mm
		Pulse duration	ms	$y = -3.56\ 10^{-5} x + 1.11$	-0.13 (0.65), 0.20	$100 < x < 1{,}250$ mm
	2000 g	Pressure	MPa	$y = 0.785\ x^{0.7584}$	- , 0.99	$100 < x < 700$ mm
		Pulse duration	ms	$y = -3\ 10^{-5}\ x + 1.465$	-0.06 (0.85), 0.16	$100 < x < 700$ mm
UTF	0.1 mm.s^{-1}	Pressure	MPa	$y = 0.005158\ x$	- , 0.99	$x < 10{,}000$ N
	1,899 N	Pulse duration	s	$y = 0.4291\ x^{-0.984}$	- , 0.99	$0.01 < x < 0.5$ mm.s^{-1}
	0.5 mm.s^{-1}	Pressure	MPa	$y = 0.005112\ x$	- , 0.99	$x < 10{,}000$ N
	3,798 N	Pulse duration	s	$y = 0.6191\ x^{-0.988}$	- , 0.99	$0.01 < x < 0.5$ mm.s^{-1}
	0.01 mm.s^{-1}	Pressure	MPa	$y = 0.005157\ x$	- , 0.99	x <10,000 N
	6,597 N	Pulse duration	s	$y = 0.8045\ x^{-0.992}$	- , 0.99	$0.01 < x < 0.5$ mm.s^{-1}
	0.05 mm.s^{-1}	Pressure	MPa	$y = 0.005160\ x$	- , 0.99	$x < 10{,}000$ N
	7,596 N	Pulse duration	s	$y = 1.0346\ x^{-0.983}$	- , 0.99	$0.01 < x < 0.5$ mm.s^{-1}

4 Single and multiple loading

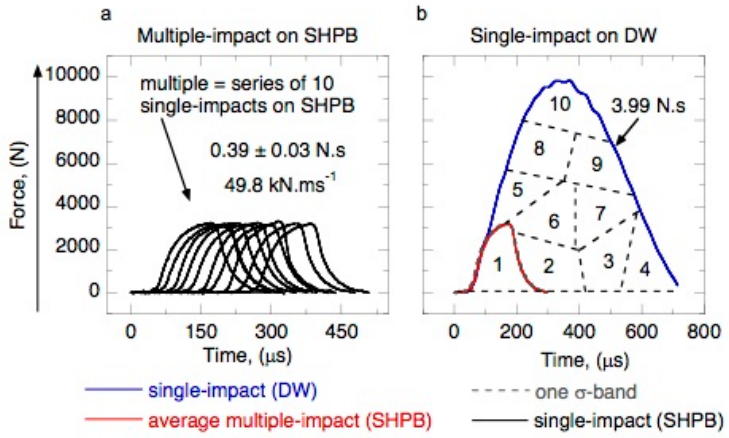


Figure 3 Single and multiple loading. (a) Series of 10 single-impact loadings on the SHPB. The pulses are slightly shifted to one another for the illustration purpose. (b) Single-impact on the DW. The single-impact DW loading is equivalent to the combination of 10 SHPB loadings in terms of delivered loading impulse and applied loading rate.

5 Calibration and validation of the *in vitro* platforms

5.1 Cell harvesting and isolation

This study was carried out under the United Kingdom's Animals (Scientific Procedures) Act of 1986 and local ethical approval from Imperial College's Animal Welfare and Ethical Review Body (AWERB) and the Home Office. Periosteum-derived mesenchymal stromal cells (PO MSCs) were harvested from the femurs of female Sprague Dawley rats (Charles River, Kent, UK) and isolated by enzymatic digestion in PBS solution containing Collagenase I (2.5 mg/ml), Collagenase II (0.7 mg/ml) and Dispase I (5 U/mL) (all reagents from Gibco) for 60 min at 37 ˚C and 5 % CO_2. The single-cell suspension was stained with primary fluorescein isothiocyanate (FITC) antibodies rat anti-mouse CD45-FITC (1/100, endothelial lineage marker), rat anti-mouse CD31-FITC (1/50, endothelial lineage marker), and rat anti-mouse HIS49-FITC (1/100, hematopoietic erythroid lineage marker) (all antibodies from Cell Signalling). The

stained cells were sorted on a flow cytometer (BD FACSAria III, BD Siosciences) to isolate PO MSCs from a heterogeneous population. The CD31-, HIS49- and CD45-positive cells were gated and excluded by negative selection

5.2 PO MSC subculture and sample preparation

PO MSCs were cultured in T225 tissue culture flasks (Dow Corning, UK) in alpha-MEM + Glutamax (ref. 32561, Gibco for Life Technologies, UK) containing 10 % (v/v) heat inactivated foetal bovine serum (FBS, Life Technologies, UK) and 1 % (v/v) penicillin-streptomycin (Pen-Strep, Invitrogen, UK) and further expanded to near-confluency (~90 %) at 37 ˚C and 5 % CO_2. For the PO MSC single cell sample preparation, a PO MSC suspension was prepared at a density of $3 \cdot 10^6$ cell.ml^{-1}. Prior to performing the mechanical loading, a final volume of 208 µl of PO MSC suspension was loaded into individual pressurization chamber using a 21-gauge needle syringe. For the PO MSCs agarose hydrogel construct preparation, a PO MSC suspension with a concentration of $6 \cdot 10^6$ cell.ml^{-1} was mixed in equal volume with a 2 % (w/v) low gelling temperature agarose solution (Type VII, Sigma, Poole, UK). The final agarose-cell suspension of $3 \cdot 10^6$ cell.ml^{-1} in 1 % (w/v) agarose hydrogel was cast into cylindrical constructs of 8.2 mm in diameter and 3.5 mm in thickness. The hydrogel constructs were incubated in 24-well tissue culture plates with 1 ml of warmed alpha-MEM + Glutamax containing 10 % (v/v) heat-inactivated FBS and 1 % (v/v) Pen-Strep. For the acellular tissue constructs, the cell suspension was replaced by equal volume of PBS.

5.3 Agarose tissue construct mechanical properties

The top part of a self aligning, ball-mounted rig is gently squeezed (F<1N) and aligned with the top platen of the UTF. The alignment is set in place using hardened epoxy-glue. For the unconfined quasi-static

testing, a well with a 20 mm diameter centre-hole was coaxially-sealed on top face of the aligned rig. The samples were tested in hydrated state.

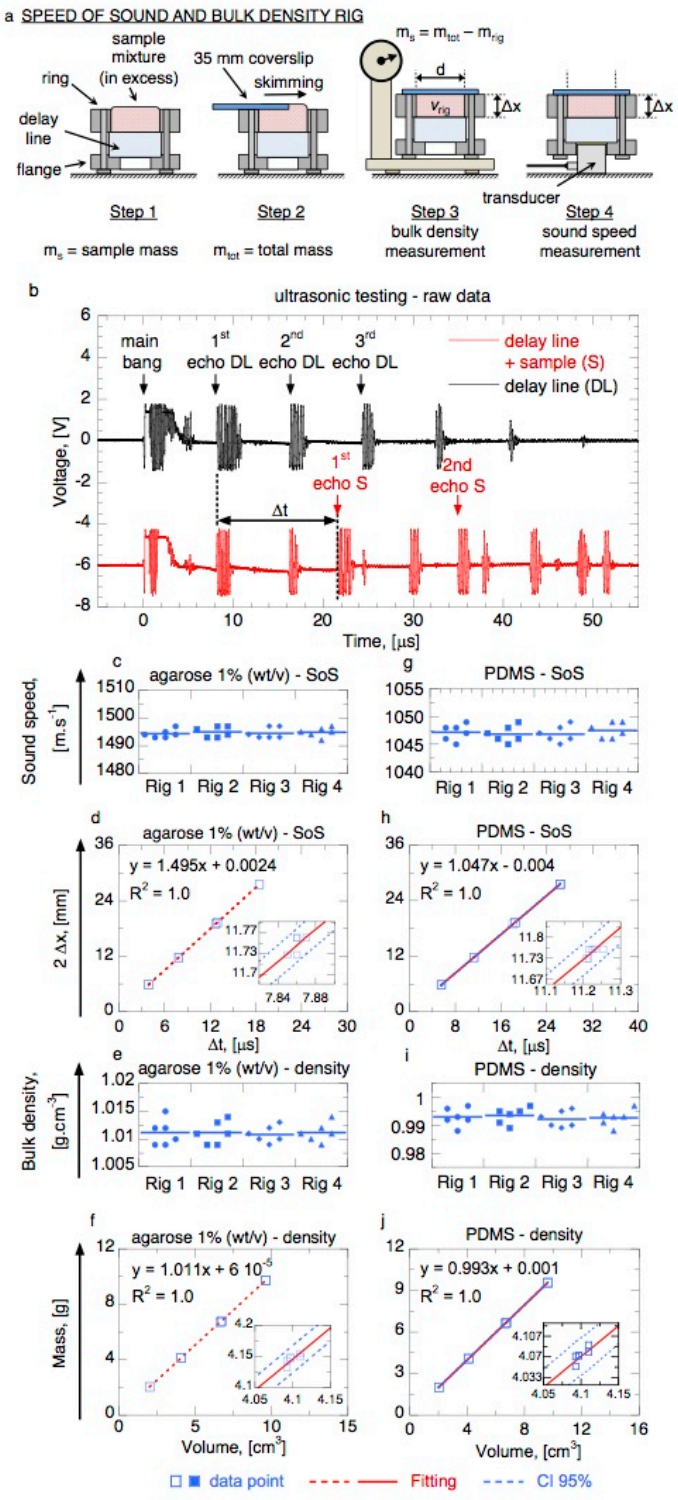


Figure 4 Initial validation of the *in vitro* platforms. (a) Correction for compliance and platen parallelism. (b) Schematic of the compression rig used for the quasi-static uniaxial compression tests of agarose hydrogel constructs. (c-e) Engineering stress-strain (σ-ε) curves of 1 % (w/v) agarose hydrogel construct before and after a compressive loading at 45 MPa peak pressure on the UTF (c), the DW (d) or the SHPB (e). (f-h), Effect a single-impact loading at 45 MPa peak pressure on the compressive elastic modulus (f), stress to failure (g) and strain to failure (h) of 1% (wt/v) agarose hydrogel constructs. Average data (n = 3) expressed as mean ± SD. (e) Effect of the pressurization chamber on the maintenance of cell health in mesenchymal stromal cells for time periods of either 5, 30, 60, 120 or 180 min. Average data (biological replicates = 4) expressed as mean ± SD.

5.4 Cell health assessment

AlamarBlue is a non-toxic, metabolic assay used to monitor metabolic activity and cell health in *in vitro* and *in vivo* models in cellular viability, test compound toxicology and biocompatibility studies. AlamarBlue assay works on the principle that the continued growth of viable cells maintains a reducing environment, while inhibition of growth maintains an oxidized environment. In brief, resazurin, which is the primary constituent of the AlamarBlue dye, appears blue in color and is non-fluorescent in its oxidized form. When it is added to the culture medium, resazurin is up taken, and reduced by the growing cells to fluorescent resorufin. Therefore, the cell health activity can be quantified by direct measurement of the fluorescence intensity of resazurin as redox indicator. AlamarBlue dye was aseptically added in an amount equal to 10 % of the culture medium volume. Protected from light, the samples were incubated at 37 ˚C and 5 % CO_2 for 6h and 2h for the hydrogel constructs and suspension samples, respectively. Thereafter, 100 µl of the reduced dye/growth medium mixture was collected from each sample well, and analysed using a SpectraMax I3x reader with SoftMax Pro 7 software (Tecan, Molecular Devices, UK) to read fluorescence emission at 590 nm, with an excitation wavelength of 560 nm. Prior to performing the assay on the mechanically loaded samples, we produced a standard curve in order to identify optimal incubation time, and the threshold to saturation conditions (**figure 5**).

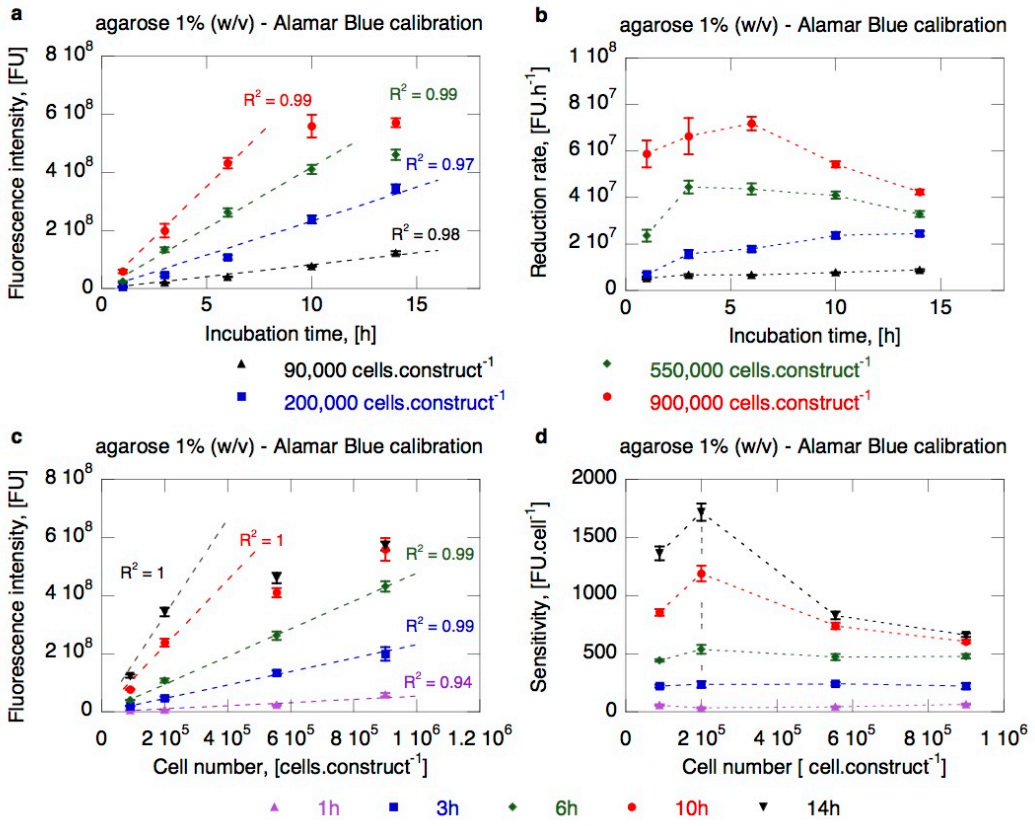


Figure 5 Alamar Blue assay calibration. (a) Average fluorescence intensity values for different incubation time and different cell encapsulation densities. (b) Rate of Alamar Blue dye reduction for different incubation time and different cell encapsulation densities. (c) Average fluorescence intensity reading values in function of the cell encapsulation densities. (d) Sensitivity of fluorescence intensity detection in function of the cell encapsulation density. Average data (biological repeats = 4) ± SD. The best linear fit is indicated in dashed line.

5.5 Cell viability assessment

The samples were incubated in a volume of 700 µl of staining solution containing 2 µM Calcein AM and 4 µM ethidium homodimer-1 (LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells, Ref L3224, Molecular Probes, Invitrogen Detection Technologies, ThermoFischer Scientific) for 30 min at 37 ˚C and 5 % CO_2. The samples were rinsed with phosphate buffer saline (PBS, Life Technologies, UK), and directly acquired to confocal microscopy imaging (Leica TCS SP5 MP/ FLIM Inverted, Leica Microsystems, UK). The parameters used were: a 488-nm laser line from an argon ion laser, a 543-nm laser line from a HeNe laser, HFT 488/543 nm, NFT 545 nm, BP 505–530 nm emission filter (Calcein

AM), and LP 585-nm emission filter (ethidium homodimer-1). Z-series images through the hydrogel constructs were collected with a dry 10X objective (**figure 6** and **figure 7**).

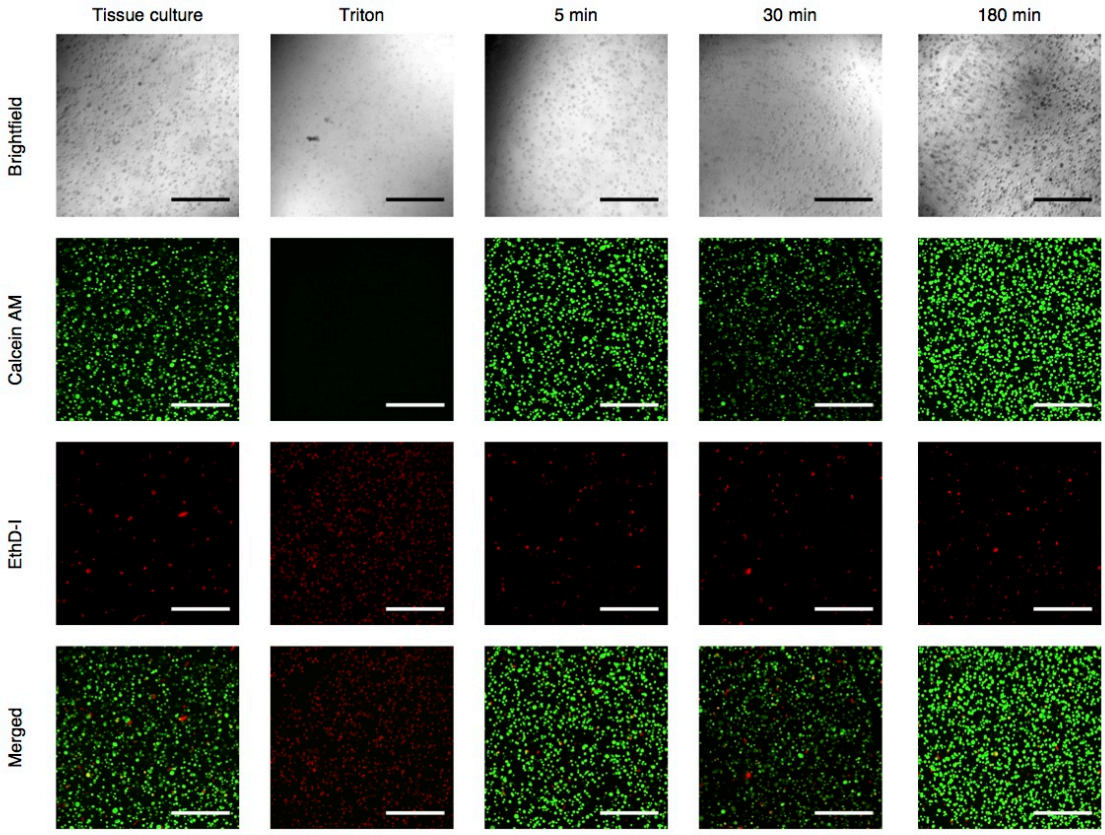


Figure 6 Validation of the platforms. Representative confocal micrographs of MSCs in agarose hydrogel after encapsulation in the pressurization chamber for time periods of 5, 30 and 180 minutes. The negative controls are MSCs incubated in standard culture conditions (in free-swelling in incubator at 37 °C and 5 %CO_2). The positive controls are MSCs treated with 1 % Triton for 30 minutes. Calcein AM: patterns of the live MSCs. EthD-1: features of the dead MSCs. Merged: fused images showing both live and dead PO MSC cells. The micrographs were taken on a confocal microscope (125 μm z-stack, Leica TCS SP5 MP/ FLIM Inverted, Leica Microsystems, UK). Scale bars = 500 μm.

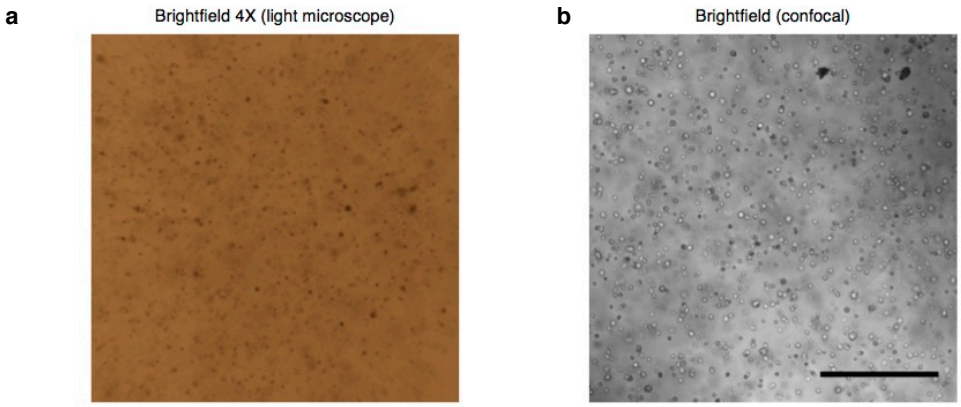


Figure 7 Representative photographs of the PO MSCs in the agarose hydrogel constructs. a, b Example of brightfield images of PO MSCs encapsulated in agarose hydogrel construct, at a final density of $3 \cdot 10^6$ cell.ml^{-1}. The images were taken on a light microscope (a) (4X, Olympus, CK40-SLP, Japan) and on a confocal microscope (b) (125 µm z-stack of brightfield, Leica TCS SP5 MP/ FLIM Inverted, Leica Microsystems, UK). Scale bar = 500 µm.

6 Mechanical compressive loading and recovery of PO MSCs.

The pressurization chamber containing the sample was interfaced with the SHPB, DW or UTF and subjected to a single-impact mechanical loading of either ~45 MPa (referred High) or ~15 MPa (referred low) nominal peak pressure (**table 3**, **table 4** and **table 5**).

Table 3 Summary of the impact conditions.

Sample type	Impact velocity, [m s^{-1}]			
	Loading	SHPB (320 mm long projectile)	DW (1050 g impactor)	UTF (-)
Single-cell suspension	low	5.62 ± 0.04	0.49 ± 0.07	(30 ± 0) ·10^{-3}
	High	19.91 ± 0.04	1.98 ± 0.01	(30 ± 0) ·10^{-3}
Agarose hydrogel constructs	low	5.61 ± 0.04	0.55 ± 0.01	(30 ± 0) ·10^{-3}
	High	19.92 ± 0.04	2.09 ± 0.01	(30 ± 0) ·10^{-3}

Table 4 Characteristics of the mechanical loading in the single-cell suspension samples. Average data (n = 4) expressed as mean ± SD

Mechanical parameter	Loading	SHPB	DW	UTF
Peak axial stress, (MPa)	low	17.22 ± 3.29	21.31 ± 1.43	20.01 ± 0.05
	High	74.82 ± 1.06	62.88 ± 2.63	70.01 ± 0.07
Average strain rate, (s^{-1})	low	600 ± 64	92 ± 13.51	0.01 ± 0.00
	High	1330 ±118	367 ± 10.87	0.01 ± 0.00
Impulse, (N.s)	low	0.23 ± 0.06	1.11 ± 0.08	8896 ± 46
	High	1.14 ± 0.03	3.17 ± 0.03	93692 ± 73
Pulse duration, (s)	low	$(362 \pm 13)\cdot 10^{-6}$	$(1.11 \pm 0.08)\cdot 10^{-3}$	14.88 ± 1.53
	High	$(339 \pm 3)\cdot 10^{-6}$	$(0.84 \pm 0.02)\ 10^{-3}$	32.28 ± 3.28
Peak axial force, (kN)	low	2181 ± 416	2699 ± 181	2534 ± 7
	High	9472 ± 134	7980 ± 329	8850 ± 27
Average loading rate, (kN ms^{-1})	low	30.78 ± 0.96	11.03 ± 0.52	$(0.71 \pm 0.02)\ 10^{-3}$
	High	95.59 ± 4.10	38.23 ± 3.29	$(0.81 \pm 0.01)\ 10^{-3}$
Peak pressure, (MPa)	low	11.25 ± 2.15	13.92 ± .093	13.07 ± 0.03
	High	48.86 ± 0.69	41.07 ± 1.72	45.72 ± 0.05

Table 5 Characteristics of the mechanical loading in the agarose hydrogel. Average data (n = 4) expressed as mean ± SD.

Mechanical parameter	Loading	SHPB	DW	UTF
Peak axial stress, (MPa)	low	20.77 ± 2.56	20.17 ± 0.98	20.01 ± 0.05
	High	68.89 ± 3.78	71.66 ± 1.03	69.97 ± 0.06
Average strain rate, (s^{-1})	low	438 ± 102	96 ± 3	0.01 ± 0.00
	High	1287 ± 81	353 ± 9	0.01 ± 0.00
Impulse, (N.s)	low	0.34 ± 0.06	1.30 ± 0.04	8324 ± 132
	High	1.10 ± 0.06	3.59 ± 0.02	89879 ± 664
Pulse duration, (s)	low	$(343 \pm 16)\cdot 10^{-6}$	$(1.11 \pm 0.08)\cdot 10^{-3}$	14.35 ± 4.71
	High	$(339 \pm 9)\cdot 10^{-6}$	$(0.84 \pm 0.02)\ 10^{-3}$	32.28 ± 3.28
Peak axial force, (kN)	low	2630 ± 324	2554 ± 124	2534 ± 7
	High	8722 ± 478	9072 ± 130	8864 ± 8
Average loading rate, (kN ms^{-1})	low	28.82 ± 7.57	9.29 ± 1.76	$(0.76 \pm 0.02)\ 10^{-3}$
	High	90.37 ± 14.64	28.76 ± 1.20	$(0.84 \pm 0.01)\ 10^{-3}$
Peak pressure, (MPa)	low	13.56 ± 1.48	13.17 ± 0.64	13.08 ± 0.03
	High	44.99 ± 2.47	46.80 ± 0.67	45.70 ± 0.04

After the compressive loading, the single-cell liquid suspension samples were transferred into individual 15 ml polypropylene centrifuge tubes (Corning, Sigma-Aldrich, UK), and centrifuged at 1,300 RPM for 2 min to form a cell pellet. PO MSCs were incubated in warmed non-selective growth media (GM) consisting of DMEM (1 $g.l^{-1}$ D-glucose, GIBCO by Life Technologies, UK) complemented with 10 % (v/v) heat-inactivated FBS and 1 % (v/v) Pen-Strep at 37 ˚C and 5 % CO_2. The cell-encapsulated hydrogel constructs were incubated into 24-well tissue culture plates pre-filled with 1 ml of warmed GM at 37 ˚C and 5 % CO_2.

7 Quantitative-reverse transcription polymerase chain reaction

The mRNA of PO MSCs was isolated with TRIzol Reagent (Invitrogen, Poole, UK) and with RNeasy mini kit (Qiagen, UK) for 3-D hydrogel constructs and the single-cell liquid suspension samples, respectively. The RT in complementary DNA (cDNA) strand was carried out using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, ref. 600084, Agilent, Stockport, UK). The q-PCR reactions were carried out in a 10 µl final volume using SYBR green (Cat. No. 4367659, ThermoFisher Scientific, UK) on a Viia7 Real-Time PCR System (Applied Biosystems, Poole, UK). The Runx2 target mRNA (Forward 5'–CGCCTCACAAACAACCACAG; Reverse 5'–TCACTGCACTGAAGAGGCTG) cycle threshold (Ct) value was normalized to the RPL13-α mRNA (Forward 5'–CTGCCGAAGATGGCGGAG; Reverse 5'–AGCGTACAACCACCACCTTT) house keeping gene Ct value [**A1,A2**]. Relative gene expression quantification was performed using the ΔΔCt method [**A3**]. All PCR reactions were performed in triplicate. All primers were purchased from Sigma.

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