

APPENDIX A

TRACTION FORCE MICROSCOPY TECHNIQUES AND ANALYSIS

A.1 *Polyacrylamide substrate preparation*

The TFM experiments presented in Chapter 5 were conducted using hydrogel elastic substrates. The hydrogel is made of polyacrylamide made by photo-crosslinking solutions of acrylamide and bis-acrylamide. As depicted the schematic in Figure 3.8, the gel is cast in two layers. The base layer consists only of polymerized polyacrylamide while the top layer contains fluorescent tracker particles and if functionalized to stimulate cell adhesion (or in the case of the experiments presented in Chapter 5, induce a phagocytic response).

A.1.1 **Coverslip activation**

The hydrogel surface is cast on top of a glass coverslip which has been treated with 3-(Trimethoxysilyl)propyl methacrylate (MaPTES) to ensure adhesion between the gel and the glass surface. In order to maximize gel thickness while preserving the ability to image TFM substrate using high numerical apertures objectives it is recommended that surfaces be made using either No. 0 or No. 1 coverslips (i.e. less than 0.16 mm thick). The glass surface is treated using the following process.

Clean coverslip

1. Sonicate coverslip in ethanol for 15 minutes
2. Sonicate in DI-H₂O for 15 minutes
3. Sonicate in 1 N NaOH solution for 30 minutes
4. Rinse with DI-H₂O, and dry with N₂

Activate with MaPTES

1. Place cleaned coverslips into glass desiccator with 100 µL of 3-(Trimethoxysilyl)propyl

methacrylate (Sigma: 440159) and 10 μ L of Triethylamine (Sigma: 471283) which acts as a catalyst.

2. Seal desiccator and pump down to approximately -25 inHg
3. Heat desiccator to 90 °C for 40 minutes, then allow to cool for 20 minutes

Coverslips may be stored under vacuum for up to 2 weeks.

A.1.2 Hydrophobic surfaces

Hydrogel substrates are formed using a liquid droplet-sandwich technique as illustrated in Figure A.2. The hydrogels are cast between the MaPTES activated coverslips and a hydrophobic glass surface. The hydrophobic glass surface is made using the following protocol.

1. Clean 35 \times 35 mm No. 1.5 coverglass using ethanol and DI-H₂O, then dry with N₂
2. Place slides in desiccator along with 100 μ L Trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (Sigma: 448931-10G)
3. Evacuate to -25 inHg
4. Heat to 100 °C for 40 minutes, then let cool for 20 minutes

Hydrophobic slides may be stored in a sealed box at room temperature and pressure indefinitely. Prior to use, rinse and dry to remove dust.

A.1.3 Acrylamide hydrogel preparation

In principle elastic substrates can be made from any transparent elastic rubber. For instance, collagen, gelatin, and PDMS (Polydimethylsiloxane) have all been used in various traction force studies [142, 87, 128]. For single-cell traction force studies polyacrylamide is an ideal substrate because it can be prepared to have a stiffness ranging from 10 – 10⁴ Pa, values compatible with measuring pico-Newton forces generated during cell activities [152, 13]. Polyacrylamide stiffness depends on the relative fraction of acrylamide monomer to bis-acrylamide crosslinker. The dependence of the Young's modulus on monomer:cross-linker ratio has been well characterized [152, 79] (see Figure A.1). A table of polyacrylamide stiffness can also be found in Plotnikov *et al.* [103].

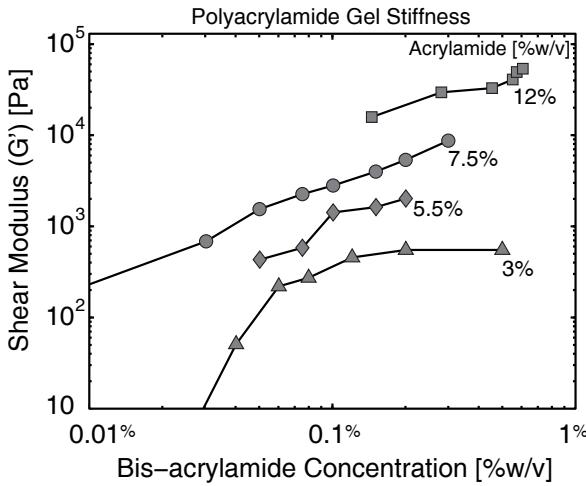


Figure A.1: Polyacrylamide stiffness versus acrylamide:bis-acrylamide ratio. Data originally presented in Yeung *et al.* [152]. Concentrations of acrylamide and bis-acrylamide are in terms of grams/mL of DI-H₂O.

The experiments presented in Chapter 5 were conducted on acrylamide surfaces with an estimated stiffness of 3000 Pa and an estimated Poisson's ratio of 0.3 (as reported in [13]). The gel hydrogel was formulated using the recipe listed in Table A.1. Prior to the hydrogel preparation, stock solutions of acrylamide (Sigma: A3553) and N,N'-Methylenebisacrylamide (Sigma: 66667) were dissolved to concentrations of 30% and 1% (in DI-H₂O), respectively. As depicted in Figure 3.8, elastic substrates consisted of two layers. The top layer included fluorescent tracker particles while the base layer was made-up of only hydrogel. Unlike many published polyacrylamide recipes ([103, 13], polymerization was not initiated using the chemical catalyst TEMED¹. Instead, polymerization was initiated using UV-light and the photo-initiator 2-hydroxy-2-methyl-propiophenone. Elastic substrates were prepared using a two-layer process (see Figure A.2).

Solution preparation

1. Before mixing sample solutions, prepare stock solutions of Acrylamide 30% w/v in H₂O and 1% w/v N,N'-methyl-bis-acrylamide in H₂O.
2. Mix two batches of polymer solution (with and without beads) following recipes

¹Typical polyacrylamide recipes use tetramethylethylenediamine (TEMED) combined with ammonium persulfate.

in Table A.1

3. Vortex gel solution to thoroughly mix
4. Desiccate gel solutions for 20 minutes to remove air bubbles

Base-layer Polymerization

1. Place hydrophobic glass on top of smooth, level, support
2. Gently pipette 30 μL of (bead-free) gel solution onto hydrophobic slide, avoid bubbles
3. Lower MaPTES treated coverslip onto gel solution
4. Expose to 365 nm UV light for 10 minutes
5. Gently submerge sample into water and use tweezer to gently remove coverslip.
Gel should release from hydrophobic surface without tearing
6. Using straight-edge razor trim gel layer to a square approximately 1 cm \times 1 cm
7. Rinse with water
8. Gently dry surface with N_2

Top-layer Polymerization

1. Pipette 10 μL droplette of gel solution with beads onto hydrophobic coverslip
2. Slowly lower droplette onto center of dry polyacrylamide base-layer, droplet should spread symmetrically upon contact

Table A.1: Acrylamide gel recipe. Solution is polymerized using UV photo-polymerization. Final stiffness is approximately 3 kPa and has an approximate Poisson's ratio of 0.3. Values in parenthesis indicate volumes use for solution containing fluorescent tracker particles.

	% by Volume	Actual Volume
30% w/v Acrylamide	5%	167 μL
1% w/v N,N'-methyl-bis-acrylamide	0.2%	200 μL
(Top Layer) 200 nm Fluorescent Beads 10% w/v washed 2 \times	(2%)	(20 μL)
2-Hydroxy-2-methyl-propiophenone	0.2%	2 μL

3. Expose to 365 nm UV light for 10 minutes
4. Gently submerge sample into water and use tweezer to gently remove coverslip.
Gel should release from hydrophobic surface without tearing
5. Using straight-edge razor trim excess gel
6. Rinse and store in water until ready to functionalize with protein

A.1.4 Polyacrylamide biofunctionalization

Polyacrylamide hydrogels are functionalized using the UV-activated cross-linking reagent Sulfo-SANPAH. For frustrated phagocytosis experiments, hydrogels are functionalized with bovine serum albumin (BSA) which acts as an antigen layer against which anti-BSA antibodies are reacted to create an immune-stimulating substrate.

BSA functionalization

1. Reconstitute Sulfo-SANPAH (Life Technologies: 22589) at 50 mg/mL in DMSO, aliquot to 40 µL and store at -80 °C until use
2. Rinse polyacrylamide gel surface with water, then shake dry
3. Dilute Sulfo-SANPAH to 2 mg/mL in DI-H₂O
4. Cover gel with Sulfo-SANPAH solution and expose to 365 nm UV light for 3 minutes
5. Wash gel several times in PBS
6. Cover slide in protein solution (1 mg/mL BSA in PBS for FP experiments), incubate 1 hour at room temperature
7. Wash gel several times in PBS

IgG Opsonization After covalently linking BSA to gel surface, substrates can be opsonized with anti-BSA IgG or serum protein

1. Place BSA functionalized coverslip face-down on 100 µL drop of 1 mg/mL anti-BSA serum protein in PBS

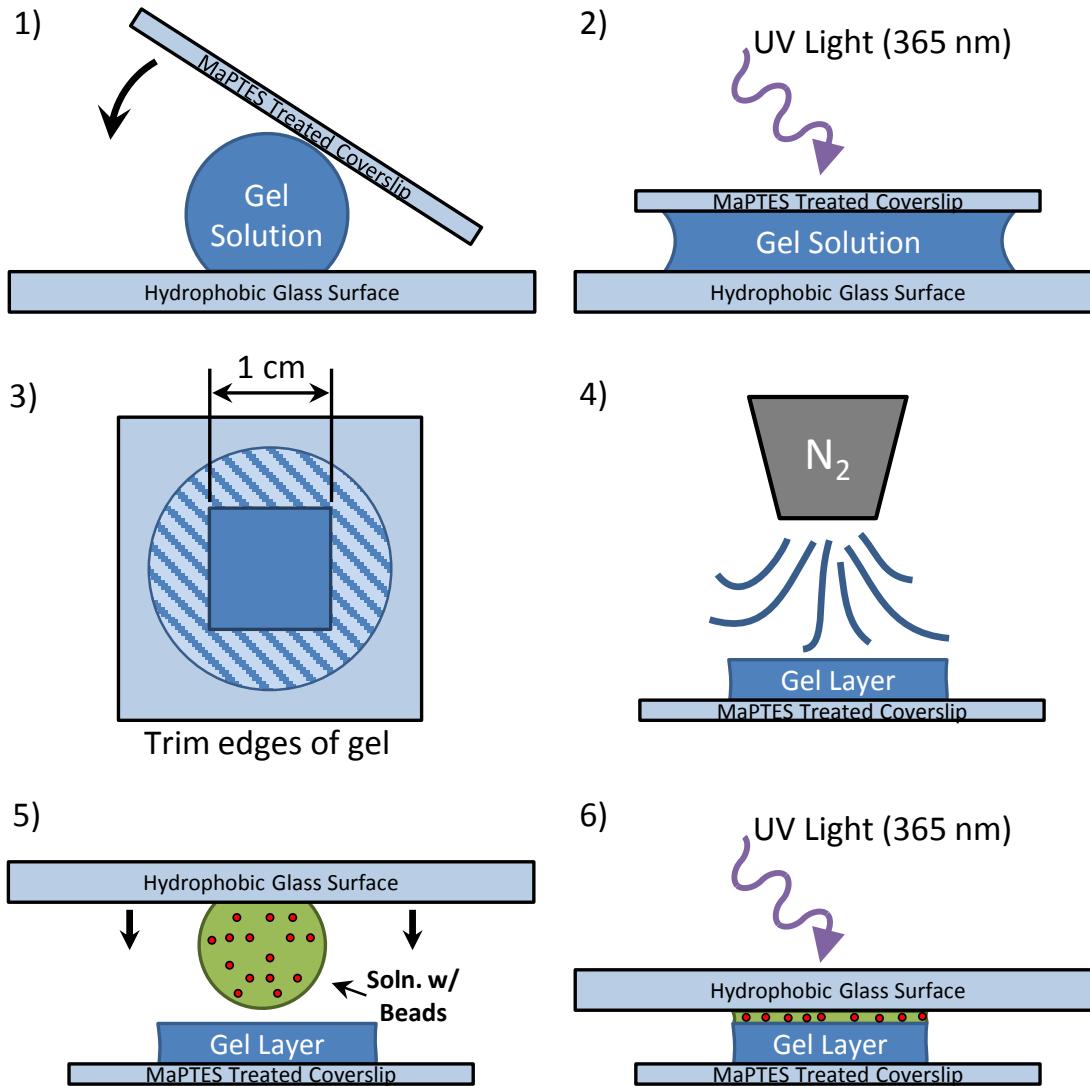


Figure A.2: TFM substrate preparation. 1) Pipette gel solution onto hydrophobic slide, cover with MaPTES activated slide. 2) Expose to UV for 10 minutes. 3) Trim gel to 1 cm square. 4) Gently dry with N_2 stream. 5) Pipette bead-containing gel solution onto hydrophobic slide, lower onto trimmed gel layer. 6) Expose to UV light for 10 minutes.

2. Incubate for 1 hour at room temperature
3. Rinse with PBS
4. Prior to use, incubate with culture media for 30 minutes at 4 °C

A.2 Measurement of stress-induced surface displacement

Live-cell frustrated phagocytosis TFM experiments are performed using a protocol similar to the standard FP protocol described in Chapter 3.1. Interaction between macrophages and the substrate were imaged using a Nikon TE-2000 microscope equipped for both DIC and fluorescent microscopy. Cells were imaged using a Nikon Plan Apo VC 60× 1.20 NA water immersion lens with a working distance of 0.27. The lens included a correction collar which was set to its maximum distance (0.19 mm) in order to maximize the working distance. For long-term imaging at physiological temperatures (37 °C), water is a poor immersion fluid because it evaporates. Instead a calibrated refractive index oil ($n_d = 1.335$) was purchased from Cargille Labs (Cargille Series: AAA). Samples were mounted on the motorized stage using a custom-built sample holder (see Figures A.4, A.3). During the course of experiments, focus was maintained using the Nikon perfect-focus system. Fluorescence microscopy was used to image motion of the tracker beads as cells engaged with the substrate.

Particle displacements were calculated using a customized particle-tracking algorithm based on code developed by Weeks and Crocker [35]. Displacement tracks were interpolated

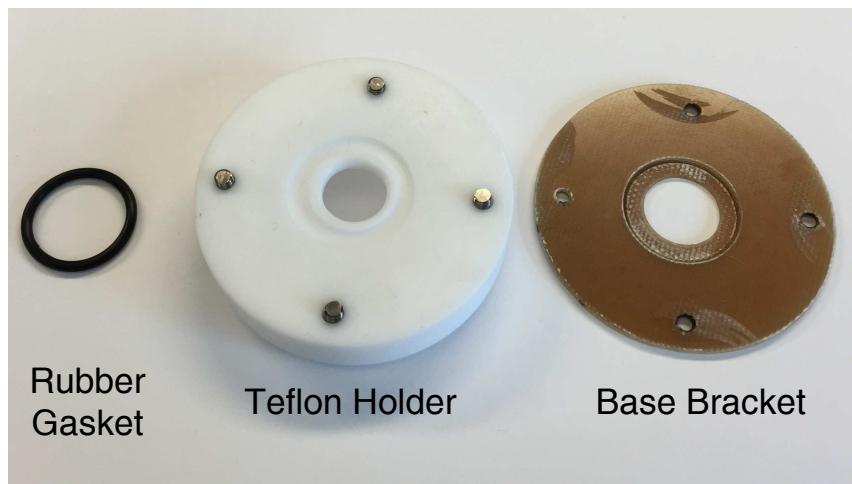


Figure A.3: TFM Sample Holder.

into a regularized grid corresponding to the approximate local- substrate displacement using the standard “scatteredInterpolant” algorithm included with MATLAB. Particle tracking errors were eliminated using filtering algorithm based on the methods presented in Westerweel and Scarano [149].

A.2.1 Calculation of traction stress

The most mathematically intensive challenge of implementing traction force microscopy is inverting the force-displacement relation for the elastic substrate. Calculations are based on the equations of equilibrium is given by the Boussinesq potential as presented in Landau-Lifshitz Theory of Elasticity [83].

The displacement within the elastic substrate caused by a point-force is given by the response-function

$$u_i = G_{ik}(x, y, z)F_k, \quad (\text{A.1})$$

with

$$G = \frac{1+\nu}{2\pi E} \begin{bmatrix} \frac{2(1-\nu)r+z}{r(r+z)} + \frac{[2r(\nu r+z)+z^2]x^2}{r^3(r+z)^2} & \frac{[2r(\nu r+z)+z^2]xy}{r^3(r+z)^2} & \frac{xz}{r^3} - \frac{(1-2\nu)x}{r(r+z)} \\ \frac{[2r(\nu r+z)+z^2]xy}{r^3(r+z)^2} & \frac{2(1-\nu)r+z}{r(r+z)} + \frac{[2r(\nu r+z)+z^2]y^2}{r^3(r+z)^2} & \frac{yz}{r^3} - \frac{(1-2\nu)y}{r(r+z)} \\ \left[\frac{1-2\nu}{r(r+z)} + \frac{z}{r^3} \right] x & \left[\frac{1-2\nu}{r(r+z)} + \frac{z}{r^3} \right] y & \frac{2(1-\nu)}{r} + \frac{z^2}{r^3} \end{bmatrix}. \quad (\text{A.2})$$

On the surface $z = 0$,

$$G = \frac{1+\nu}{\pi E} \cdot \frac{1}{r^3} \begin{bmatrix} (1-\nu)r^2 + \nu x^2 & \nu xy & -(1-2\nu)rx \\ \nu xy & (1-\nu)r^2 + \nu y^2 & -(1-2\nu)rx \\ \frac{1-2\nu}{2}xr & \frac{1-2\nu}{2}yr & (1-\nu)r^2 \end{bmatrix}. \quad (\text{A.3})$$

Assuming no normal forces and ignoring normal displacements

$$G(x, y) = \frac{1+\nu}{\pi E} \cdot \frac{1}{r^3} \begin{bmatrix} (1-\nu(r^2 + \nu x^2) & vxy \\ vxy & (1-\nu(r^2 + \nu y^2) \end{bmatrix}. \quad (\text{A.4})$$

For a distribution of stress applied to the surface

$$u_i(x, y) = \int \int G_{ij}(x - x', y, y') T_j(x', y') dx' dy' = G_{ij} * I_j. \quad (\text{A.5})$$

Taking the Fourier-transform

$$\tilde{u}_i(k_x, k_y) = \mathcal{F}[G_{ij} * T_j] = \tilde{G}_{ij}(k_x, k_y) \cdot \tilde{T}_j(k_x, k_y). \quad (\text{A.6})$$

Consequently the traction force can be solved by inverting the linear equation in Fourier-space and then taking the inverse Fourier transform. The Fourier-space response tensor is

$$\tilde{G} = \frac{2(1+\nu)}{E} \cdot \frac{1}{k^3} \begin{bmatrix} k^2 - \nu k_x^2 & -\nu k_x k_y \\ -\nu k_x k_y & k^2 - \nu k_y^2 \end{bmatrix}. \quad (\text{A.7})$$

If you assume that the displacement field is without noise we can solve for \mathbf{T} by simple inversion

$$\tilde{\mathbf{T}} = \tilde{G}^{-1} \cdot \tilde{\mathbf{u}} \quad (\text{A.8})$$

$$\tilde{G}^{-1} = \frac{E}{2(1+\nu)} \cdot \frac{1}{k(1-\nu^2)} \cdot \begin{bmatrix} k^2 - \nu k_y^2 & \nu k_x k_y \\ \nu k_x k_y & k^2 - \nu k_x^2 \end{bmatrix}. \quad (\text{A.9})$$

A.2.2 Fourier Transform Traction Force Cytometry (FTTC)

Using the relationship defined in eq A.8, the stress-field generated by a cell can be calculated using a Fourier-method termed Fourier Transform Traction Force Cytometry (FTTC) [25]. First, the frame-by-frame 2-D Fourier-transform of the regularized displacement field is calculated. Next, this Fourier-space displacement field is multiplied by the inverse Boussinesq potential calculated in eq A.9, yielding the Fourier-transform of the traction stress. Finally, the real-space traction stress is calculated by taking the inverse Fourier-transforms. The result is a stress-field defined at regular intervals. An example can be seen in Figure 5.2.

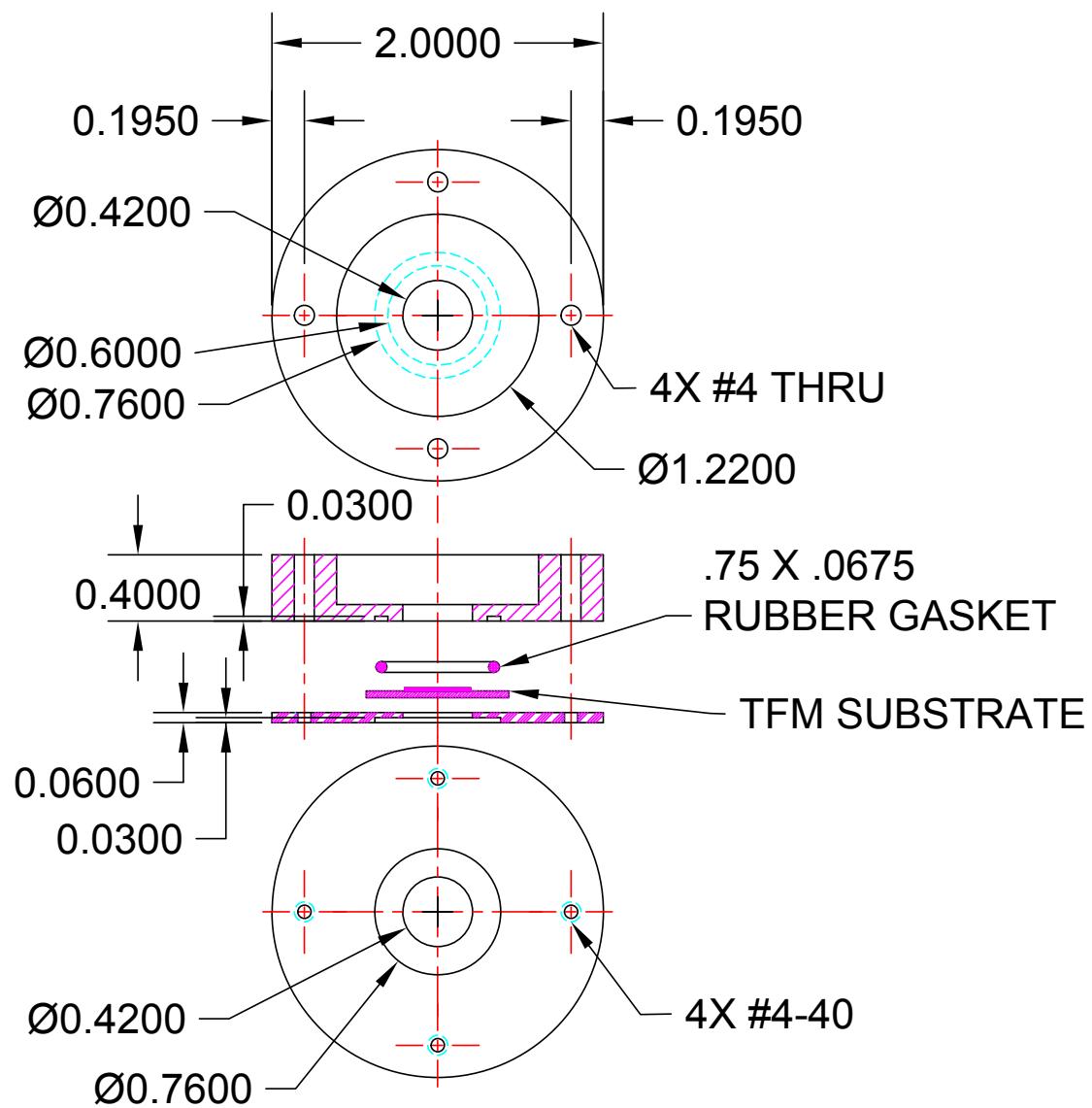


Figure A.4: TFM Sample Holder Schematic. All dimensions are in inches.

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