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Hydrogel Microparticles as Force Sensors: Synthesis and Application in Phagocytosis Studies

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Hydrogel Microparticles

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Abstract

We provide a protocol for the synthesis of tuneable hydrogel microparticles (HMPs), and their application to measure forces during phagocytosis by macrophages. A specific type of deformabale acrylamide-based HMP, DAAM-particles, are synthesized in batch using a membrane emulsification approach. They then are conjugated with both biologically active molecules and fluorescent labels in a one-pot functionalization procedure. Macrophages are incubated with the functionalized DAAM-particles and imaged by regular confocal microscopy. With a custom image analysis strategy, local DAAM-particle deformations can be quantified with superresolution accuracy (< 50 nm) in 3D throughout phagocytosis. Finally, normal and shear forces can be inferred from the 3D shape of DAAM-particles, revealing the spatial distribution of cellular forces. The entire protocol takes 2-3 days and requires only basic expertise in mammalian cell culture and fluorescence microscopy, and minimal specialized equipment. This protocol can be adapted for studying several other cellular processes, and thereby broadly applied in mechanobiology and immune cell biology.

Materials

Section 1. Deformable Acrylamide co-Acrylic acid Microparticle (DAAM-particle) synthesis

Equipment:

- SPG micro-kit module (SPG Technology, MN-20)
- Tubular hydrophobic Shirasu porous glass (SPG) membranes of 20 mm length, 10 mm outer diameter and with pore size (diameter) 0.5 – 3 µm, (SPG Technology)
- (Sealable Jacketed) flask
- PTFE-lined septa
- Sonication Bath
- Pump or house vacuum line
- N2 line
- Water Bath
- Magnetic steering plate
- 2x Büchner flask
- 2x Sleeve typed septum stopper
- Shut-off valve
- Pressure gauge (4 – 100 kPa)
- pressure regulator (4 – 100 kPa)
- Non-coring tip needles
- Cannula
- Lab stand
- Centrifuge
- Glass tubes with lids for centrifugation
- Phase contrast microscope with a 40x objective lens
- Microscope glass slides and cover slips
- Hemacytometer

Software:

- ImageJ (FIJI),

Chemicals:

- Span (Polysorbate) 80 (Sigma-Aldrich S6760)
- Hexanes, mixture of isomers (>99% purity)
- Sodium Hydroxide (NaOH)
- MilliQ water (MQ water, 18.2 Ω),
- Acrylic acid (Aac, 98% extra pure)
- N, N'- methylenebisacrylamide (BIS)
- Mono-acrylamide (Aam)
- tetramethyl-ethylenediamine (TEMED) (>99% purity)
- 2,2'-Azobisiobutyrontirile (AIBN)
- n-Dodecane (99% purity)

- Ethanol (99% purity)
- Phosphate-buffered saline (PBS)
- Tween (polysorbate) 20, (Tween buffer solutions should be used within one month of preparation),
- 3-(N-morpholino) propane sulfonic acid (MOPS) sodium salt

Reagent setup:

- 1x Phosphate-buffered saline, PBS (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4)
- 300 mM MOPS

Section 2. DAAM-particle functionalization

Equipment:

- Tabletop centrifuge
- End to end rotator

Chemicals:

- DAAM-particles synthesized in section 1
- N-(3-dimethylaminopropyl)-N’-ethylcabodiimidehydrochloride (EDC, Sigma-Aldrich, E7750)
- N-hydroxysuccimide (NHS,Thermo Scientific, A10312.22)
- MES sodium salt
- Ethanolamine (purity > 99%)
- tris(hydroxymethyl)aminomethane (Tris-Cl)
- Tween (polysorbate) 20
- Sodium azide
- Sodium chloride (NaCl)
- Bovine Serum Albumin (BSA)
- anti-BSA rabbit IgG (MP Biomedicals, 0865111)
- 5/6-TAMRA cadaverine

Reagent setup:

- 10% tween 20
- PBS (pH 7.4)
- PBS + 0.1 % tween 20 (pH 7.4) (PBS-T, pH7.4)
- 0.1x PBS (pH6) +0.2% tween 20, (PBS-T, pH6)
- 2x PBS (pH8.5)
- Activation buffer: 100 mM 2-(N-morpholino) ethane sulfonic acid (MES) buffer (prepared from MES sodium salt) and 200 mM NaCl (pH 6.0). (Store at 4C for ~6 months max)
- Tris buffer: 300 mM Tris (pH 9.0) + 300 mM NaCl
- 5/6-TAMRA cadaverine at 20 mM concentration in PBS (pH 7.4) (Store at -20C)
- 0.5 M sodium azide

Section 3: Phagocytic assay

Equipment:
- Biosafety cabinet
- Large capacity centrifuge (for well plates) (300 g)
- Incubator (37 °C)
- 24-well glass bottom plate (Cellvis, P24–1.5H-N)

Cell culture:
- J774A.1 (ATCC, TIB-67) cell line
- Dulbecco's Modified Eagle Medium (DMEM, Gibco CAT No. 42430-025)
- heat-inactivated Fetal Bovine Serum (hiFBS, Thermofisher CAT No. 10270106),
- 1% antibiotic-antimycotics (Thermofisher CAT No. 15240-062)

Chemicals:
- Formaldehyde solution (J.T. Baker, 2106–01)
- Alexa Fluor-647 donkey anti-rabbit IgG (ThermoFisher, CAT. No. A-31573),
- Triton x-100
- Alexa Fluor 488-Phalloidin (ThermoFisher, CAT No. A12379)
- PBS (pH 7.4)

Reagent setup:
- DMEM full: (Dulbecco's Modified Eagle Medium (DMEM, Gibco CAT No. 42430-025) supplemented with 10% heat-inactivated Fetal Bovine Serum (hiFBS, Thermofisher CAT No. 10270106), and 1% antibiotic-antimycotics (Thermofisher CAT No. 15240-062))
- 4% Formaldehyde solution dissolved in 1x PBS (pH 7.4),
- 0.2% Triton X-100,

Software:
- Matlab
- Python

Section 4: Imaging & data analysis

Equipment:
- Point-scanning or spinning disc confocal microscope (excitation lasers 488, 561 and 647 nm) equipped with a high magnification (60 - 100x), high numerical aperture (NA (~1.4)) oil objective and a piezo z-stage with a plate insert

Software:

- Matlab
- Python

Safety warnings

⚠️ Multiple chemicals used in this protocol, including n-dodecane, hexanes, acrylamide, acrylic acid, sodium azide, TEMED, EDC, AIBN, Triton X-100, sodium hydroxide, and formaldehyde solution, present health hazards, are irritants, and/or are flammable. Please use and wear appropriate PPE and refer to the safety data sheets (SDS) provided by the manufacturers, and abide local safety and environmental regulations, including for disposal.

⚠️ Perform DAAM-particle synthesis in a fume hood for safety.

⚠️ AIBN can be explosive on shock. Handle it carefully.

Deformable Acrylamide co-Acrylic acid Microparticle (DAAM-particle) synthesis

1d

1 Prepare the extrusion equipment

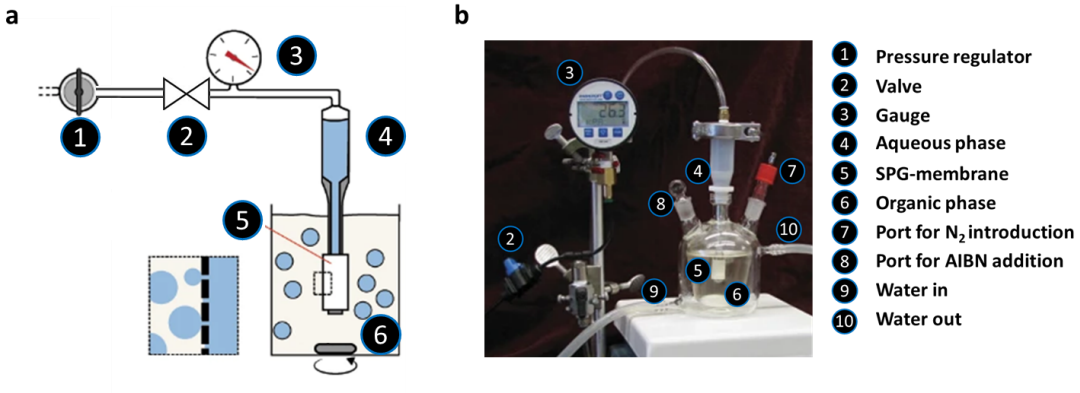

Figure 1: Overview of the setup for DAAM-particle synthesis. (a) Schematic and (b) photograph of the essential components. The complete experimental setup involves adjusting and measuring pressure using the pressure regulator (1),valve (2) and gauge (3). The aqueous phase, containing the gel mixture, is added to the SPG module reservoir (4) and extruded through the SPG membrane (5) into a constantly spinning organic phase (6) placed on a magnetic steering plate. The process is conducted under a nitrogen atmosphere, which is introduced via one of the ports of the flask (7). After extrusion is complete, polymerization inside droplets is triggered by addition of AIBN (8). The jacketed flask hooked up to a water bath, with inflow (9) and outflow (10) of water, provides temperature control. Adapted from Vorselen et al., 2020.

1.1 Establish N_2 flow from a compressed gas cylinder or a house N_2 line. Insert a pressure regulator, shut-off valve and the pressure gauge in the indicated sequence (Fig. 1). Ensure an air-tight fit of the N_2 line between the shut-off valve and the top of the SPG module.

1.2 Flask selection: Check if the flask ensures a proper fit of the SPG module. Allow N_2 atmosphere regulation by creating a closed system. Allow addition of the polymerization initiator (AIBN) and pressure release during the process. To simplify workflow, a jacketed flask is recommended but not essential.

Note

Suggestion 1: Any sealable flasks with at least one opening (neck) compatible with the SPG module can be used. However, it is convenient to have more inlets to simplify workflow for pressure regulation and AIBN addition. If a non-jacketed flask is used, the polymerization reaction can be carried out by placing the flask directly in the water bath.

1.3 Set up a temperature control system: Position a water bath next to the set up (without turning it on).

2 SPG Membrane preparation

2.1 Choose a SPG membrane with a pore size to synthesize DAAM-particles of your desired diameter (Fig. 2).

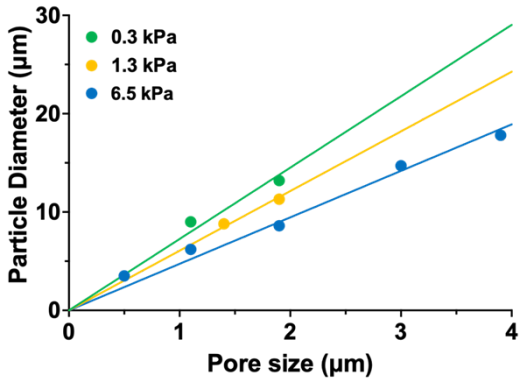

Figure 2: Correlation between SPG pore diameter and particle diameter for DAAM-particles with a Young's modulus of 6.5 kPa (blue, y = 4.3x +1.3), 1.3 kPa (yellow, y=5x + 1.8) & 0.3 kPa (green, y = 5.6x + 3.2). For the exact particle compositions, see table 1. Variations in total acrylamide and crosslinker concentration will influence hydrogel swelling, leading to changes in particle size. Adapted from Vorselen et al., 2020.

2.2 Slowly immerse the membrane in n-dodecane. Continuously allow the membrane to wet itself through capillary forces before submerging it further.

2.3 Degas the membrane by sonication under vacuum by placing the membrane in a sonication bath while inducing and maintaining a vacuum until no observable bubbles are formed (~ 00:05:00).

⚠ **Critical step:** Inadequate degassing of the membrane can lead to unsuccessful extrusion, as the presence of air within the membrane pores can obstruct the initiation of the extrusion process.

2.4 Keep the membrane in the n-dodecane until further use.

3 Preparation of the organic and aqueous phase.

3.1 Prepare a 3% v/v solution of Span 80 in hexanes with a total volume of 150 mL.

3.2 Stir the organic phase until the Span 80 is completely dissolved.

3.3 Transfer the organic phase to the (sealable) flask used for polymerization and stir the organic phase 300 rpm under a nitrogen (N_2) atmosphere for at least 00:20:00

3.4 In the meantime, mix the components of the aqueous phase. Tested DAAM-particle compositions with specific properties of the resulting particles are given in Table 1.

Table 1. Overview of parameters for synthesizing diverse DAAM-particles with different Young's modulus (0.3 – 6.5 kPa) and a (cell-sized) diameter of 9 µm. Typically, a total volume of 12 ml is made.

	A	B	C	D
		Soft (0.3 kPa) DAAM-particles	Intermediate (1.3 kPa) DAAM-particles	Stiff (6.5 kPa) DAAM-particles
	Membrane pore size (diameter)	1.1 µm	1.4 µm	1.9 µm
	Mono-Acrylamide	89.7 mg/ml	89.3 mg/ml	87.7 mg/ml
	Bisacrylamide	0.032 mg/ml	0.67 mg/ml	2.34 mg/ml
	Acrylic acid	10 mg/ml	10 mg/ml	10 mg/ml
	MOPS	150 mM	150 mM	150 mM
	NaOH	150 mM	150 mM	150 mM
	TEMED	0.3% v/v	0.3% v/v	0.3% v/v
	MQ Water	As needed to reach the total volume	As needed to reach the total volume	As needed to reach the total volume

Note

Suggestion 2: By changing the composition of the acrylamide mixture, particle rigidity can be tuned. The total amount of acrylic compounds (Acrylamide + Bisacrylamide + Acrylic acid) can be kept constant at 100 mg/mL for particles up to at least 20 kPa. Within this range, changing the ratio of bisacrylamide and mono-acrylamide is sufficient to obtain DAAM-particles with different rigidities (Table 1). The concentration of MOPS, Acrylic acid, NaOH and TEMED are independent of the desired rigidity. Synthesizing DAAM-particles with a different desired rigidity then those stated in table 1 involves experimental optimization of the acrylamide mixture, as precisely predicting the Young's modulus from the composition proves challenging. It should be noted that the observed correlation between crosslinker concentration and rigidity on flat bulk acrylamide hydrogels seems to differ from DAAM-particles generated using this protocol.

3.5 Place the aqueous phase under a vacuum for 00:15:00 .

⚠ **Critical step:** Insufficient degassing of the aqueous phase can result in poor particle polymerization.

3.6 Put the aqueous phase under a N_2 atmosphere (00:05:00), ensuring an oxygen-free environment without the need for continuous nitrogen flow.

4 Setup the SPG module and determine the critical pressure (P_c)

4.1 Assemble the SPG internal pressure micro kit according to the manufacturer instructions.

4.2 Take the SPG membrane out of the dodecane and attach it to the internal pressure micro kit extruder, now named SPG module.

4.3 Immediately immerse the module slowly into the constantly steering organic phase and ensure a closed system.

4.4 Inject 10 mL of the acrylamide mixture (aqueous phase) into the SPG module reservoir under nitrogen pressure using a cannula.

Note

Suggestion 3: A small amount (< 2 mL) of acrylamide mixture will be left. Polymerizing this access acrylamide using APS (Ammonium persulfate) can be used to check for issues with polymerization.

4.5 Attach the nitrogen line to the SPG module.

4.6 Determine critical pressure (P_c) following the steps in the note below. If (re)using a membrane with identical pore size to a previously used membrane, these steps can be skipped and the previously determined value can be used instead.

Note

Use Fig. 3 as a reference to obtain an initial estimate of the critical pressure (P_c) in kPa for the selected membrane pore size (pore size diameter D)

- Set the pressure below the known (from previous attempts) or estimated critical pressure.
- Open the shut-off valve to pressurize the aqueous phase in the SPG module reservoir.
- Close the shut-off valve and monitor if the pressure is constant over a 3-minute interval (drop < 0.2 kPa). Constant pressure indicates that the set extrusion pressure is not sufficient to extrude the gel mixture through the membrane.
- If no pressure drop is observed, open the shut-off valve and and increase the pressure by 1 - 2 kPa".
- Repeat step 2.3 and 2.4 until a pressure drop is observed (>0.2 kPa), indicating that the aqueous phase is passing through the membrane.

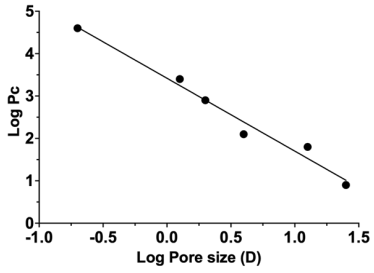

Figure 3: Correlation between the membrane pore size (diameter, D, µm) and the critical pressure (P_c, kPa) for acrylamide gel mixtures in hexanes using 3% (v/v) span 80 as surfactant. Shown on a log-scale to illustrate the linear relationship. P_c = exp(-1.7*ln(D)+3.4).

⚠ **Critical step**: Identifying the critical pressure and maintaining constant pressure (1.1 – 1.5 times P_c) during extrusion is crucial for establishing monodisperse emulsions. Pressure below P_c preclude flow, and too high pressure (typically 2-3 times P_c) results in highly polydisperse particles. If flow is observed below P_c, there is potentially a leakage between the shaft of the extrusion kit and the membrane, and the aqueous phose is not passing through the membrane, also leading to polydisperse particles.

5 Emulsification and polymerization

5.1 After the critical pressure is determined, open the valve and keep the pressure constant during the extrusion of the entire aqueous phase while continuously steering the emulsion (check periodically, about once every hour, to ensure a continuous flow). When extrusion is completed (no gel mixture in SPG module), close the nitrogen flow. Typical time 03:00:00

Note

Troubleshooting 1: If issues with extrusion arise, such as low or irregular speed, consider decreasing the percentage of Span 80 in the formulation. A minimum of 1% Span 80 is recommended for beads with uniform size.

5.2 Carefully remove the SPG module from the emulsion and close the flask containing the emulsion securely.

Note

Troubleshooting 2: To verify the success of the extrusion process, before starting the polymerization, examine a small sample of the emulsion under a microscope in a closed glass vial. Ensure the droplets appear monodisperse. They will often adopt a hexagonal close-packing configuration. If the droplets are not monodispersed, it is advisable to restart the procedure.

5.3 Remove the membrane from the module and place it in n-dodecane. Sonicate it for 00:05:00 . Then store the membrane in fresh dodecane.

5.4 Set the water bath to 62 °C and start the flow.

Note

Troubleshooting 3: Deviation from the recommended temperature during polymerization can impact the success of the polymerization process. If polymerization issues are occurring, check the emulsion temperature before addition of AIBN using a thermometer. The temperature of the emulsion should be ~ 60 °C .

5.5 Weigh out 225 mg of AIBN (1.5 mg/ml final concentration) and add it to the flask. Incubate for 03:00:00 for the polymerization to complete. Periodically release any excess pressure by insertion of a needle. After 3 hours, let the emulsion cool down to room temperature.

⏸ **Pause Point:** Polymerized particles are stable. They can be washed and collected the next day.

Note

Troubleshooting 4: To assess the success of the polymerization step, take a small volume from the emulsion and add ethanol, which will dehydrate the DAAM-particles. If the solution turns transparent with no visible white precipitate, this suggests unsuccessful polymerization. Conversely, if white precipitate is formed, it indicates successful polymerization. Furthermore, directly after the heating step, a small volume of the emulsion can be evaluated under the microscope to assess droplet monodispersity. If the emulsion destabilizes after heating, it indicates an issue with emulsion stability, possibly stemming from a lower than expected salt concentration.

5.6 Wash the particles three times with an excess of hexanes by spinning down at 300 x g, 00:05:00 .

5.7 Wash the particle once with an excess of ethanol.

5.8 Resuspend the particles in 80 mL of PBS and wait approximately 00:30:00 for the particles to rehydrate.

5.9 Perform two additional washes with PBS by spinning down at 16000 x g, 00:05:00 .

5.10 Add sodium azide to a final concentration of [M] 5 millimolar (mM) and store the DAAM-particles at 4 °C.

Pause point: polymerized particles are stable for long term storage (at least 1 year) in PBS

6 Measuring DAAM-particles properties.

6.1 Concentration

- Dilute the DAAM-particles 100-fold in PBS (pH 7.4) containing 0.1% Tween 20.
- Utilize a hematocytometer and a phase contrast microscope to determine particle concentration.

6.2 Size

- Dilute the DAAM-particles 10-fold in PBS, pH 7.4 and put it on a microscope glass slide with coverslip.
- Acquire phase contrast microscopy images of DAAM-particles +(~ 500) with a 40x objective lens.
- Analyze the acquired images in IMAGEJ (see note).

Note

1. Preprocess the images by applying (process ->) “smooth” two times.
2. Use (process ->) “Find edges” to obtain particle outlines.
3. Use (Image -> adjust ->) “Threshold” to binarize the images.
4. Use (Process -> Binary ->) “Fill holes” within the binary particles.
5. Employ the (Process -> Binary ->) “Watershed” algorithm to separate adjacent particles.
6. Use (Analyze ->) “Analyze Particles” to determine their areas. Image artifacts and small contaminants can be excluded from analysis by setting a minimum area (depending on the particle size) and sphericity (~0.9).

- Calculate the diameter of the microparticles from their respective areas ($D = \sqrt{(4A/\pi)}$ where D is the diameter and A the obtained area.
- Calculate the coefficient of variation ($CV = STD(D)/D$)) to evaluate particle batch monodispersity. Where $STD(D)$ is the standard deviation and D the mean diameter. CV should be ~0.1.
- Calculate the volume/volume (v/v) percentage of the DAAM-particles based on the volume occupied by the spheres (DAAM-particles), multiplied by the concentration of the DAAM-particles. ($v/v\% = C * (4/3\pi * (D/2)^3)/10^{10}$)where C is concentration DAAM-particles/ml, and D the diameter in µm.

DAAM-particle functionalization

4h

7 Dilute DAAM-particles to a concentration of 5% v/v in PBS (pH 7.4) .

Note

Suggestion 4: The functionalization of DAAM-particles can be done in parallel for multiple conditions and types.

7.1 Take 0.5 mL from the 5% v/v DAAM-particles.

7.2 Wash the particles twice in 0.5 mL activation buffer by spinning down for 10000 x g, 00:01:00 and thoroughly mixing (by vortexing) in between.

Note

Suggestion 5: The time and speed of the centrifugation step depend on the rigidity and size of the DAAM-particles (Table 2). Using higher than necessary spin speeds could result in DAAM-particle clustering.

Table 2. Recommended spin speeds for ~9 µm diameter DAAM-particles.

	A	B	C	D
		Soft (0.3 kPa) DAAM-particles	Intermediate (1.3 kPa) DAAM-particles	Stiff (6.5 kPa) DAAM-particles
	Time	2 min	1 min	30 s
	Centrifugation speed (x g)	16000	10000	1000

7.3 Resuspend the washed DAAM-particles in 300 µL activation buffer.

7.4 Add 5 µL of 10% Tween to the DAAM-particles (0.1 % v/v final concentration)

7.5 Add 100 µL of [M] 200 mg/mL EDC, freshly dissolved in activation buffer, to the DAAM-particles and thoroughly mix the solution.

7.6 Add 100 µL of [M] 100 mg/mL NHS, freshly dissolved in activation buffer, to the DAAM-particles and thoroughly mix the solution.

7.7 Incubate in an end-to-end rotator at Room temperature for 00:15:00. In the meantime, prepare a new tube with 50 µL of 100 mg/ml BSA solution.

⚠ **Critical step:** During all subsequent resuspension steps (7.8-7.10), vigorously vortex the particles to prevent particle aggregation. When continuing with clustered particles, these will likely be covalently cross-linked and impossible to break up later. Of note, sonication is not recommended as it can break the internal structure of soft DAAM-particles.

7.8 After incubation, wash the DAAM-particles three times with PBS-T, pH 6.

Note

Suggestion 6: The functionalization steps can be replaced with alternative proteins and carboxyl-reactive-fluorophores (see accompanying Plos One manuscript, Fig. 3). Alternative carboxyl-reactive fluorescent dyes include Rhodamine-cadaverine, Alexa488-cadaverine, Alexa647-cadaverine, Lissamine Rhodamine B-ethylenediamine and other amino-derived photostable dyes. For performing microparticle traction force microscopy, dyes with good photostability are required. If choosing different dyes, ensure that they are compatible (minimal spectral overlap) with cellular staining performed during the phagocytic assay.

7.9 Following the final wash, resuspend the DAAM-particles in 225 µL PBS-T, pH 6 and mix the dispersion thoroughly.

7.10 Add 225 µL of 2x PBS (pH 8.5) to the resuspended DAAM-particles. Mix briefly by pipetting up and down and immediately (one tube at a time) transfer the particles to the BSA solution.

7.11 Incubate at Room temperature for 01:00:00 using an end-to-end rotator. In the meantime, prepare a new tube with 5/6-TAMRA cadaverine ([M] 0.2 millimolar (mM) final concentration).

7.12 Add the entire DAAM-particles-BSA solution to the tube containing 5/6-TAMRA cadaverine, mix vigorously, and incubate at Room temperature for 00:30:00 using an end-to-end rotator.

7.13 Prepare 250 µL blocking buffer by adding [M] 300 millimolar (mM) ethanolamine to the Tris buffer.

7.14 Add 250 µL of the blocking buffer to the DAAM-particles solution and incubate at Room temperature for 00:30:00 using an end-to-end rotator.

7.15 Wash the functionalized DAAM-particles 3 times with PBS-T, pH7.4.

Pause point: After this step, particles can be stored for at least 1 month at 4 °C with [M] 5 millimolar (mM) sodium azide.

Note

Troubleshooting 5: The final concentration of DAAM-particles may fall below 5% solids due to losses or clustering during wash steps. It is recommended to determine the DAAM-particle concentration and readjust the particle suspension to the preferred concentration of 5% v/v.

8 IgG opsonization

8.1 Add anti-BSA antibody to DAAM-particles in PBS-T (pH 7.4) to final antibody concentration of 0.1 mg/ml .

8.2 Incubate at Room temperature for 01:00:00 using an end-to-end rotator.

8.3 Wash the functionalized DAAM-particles 3 times with 1X PBS (pH 7.4)

8.4 Use directly or store at 4°C in 1x PBS (pH 7.4) with 5mM sodium azide.

⏸ **Pause Point:** after this step, particles can be stored at 4°C for at least 2 weeks with 5 mM sodium azide.

Phagocytic assay

4h

9 **Cell seeding**

9.1 Maintain J774A.1 cells in DMEM full (Prepare hiFBS by heating FBS at 56 °C for 00:30:00).

9.2 Seed 0.75 × 10^5 J774A.1 cells per well in a 24-well glass-bottom plate and incubate Overnight .

Note

Suggestion 7: If desired, adapt seeding density for other cell types and plate formats (scale cell amounts by well surface area).

10 **Phagocytosis assay**

10.1 Centrifuge DAAM-particles and resuspend them in DMEM full at 2.5 × 10^6 DAAM-particles per mL.

10.2 Replace the medium in each well with 200 µL DMEM full containing DAAM-particles (5 × 10^5 DAAM-particles per well).

10.3 Centrifuge the plate for 300 x g, 00:01:00 to promote synchronized interactions with cells.

10.4 Incubate the plate at 37 °C for 00:05:00 - 00:30:00 .

11 **Immunostaining**

Note

Suggestion 8: These staining steps can be complemented or replaced with labelling of other membrane or cytoplasmic proteins of interest.

11.1 Fix the cells by removing the medium and adding 200 µL of 4% formaldehyde in PBS (pH 7.4) per well.

11.2 Wash wells three times with 200 µL PBS (pH 7.4) to remove fixative solution and unattached DAAM-particles by gently pipetting up and down.

11.3 To stain the exposed DAAM-particles surface, replace the medium in the wells with 200 µL of PBS (pH 7.4) containing 4 µg/ml Alexa Fluor-647 donkey anti-rabbit IgG and incubate for 00:30:00 .

11.4 Wash the wells with 500 µL PBS (pH 7.4) three times.

11.5 To permeabilize cells, replace the medium in the cells with 200 µL of PBS (pH 7.4) containing 0.2% Triton X-100 solution.

11.6 Incubate the plate for 00:10:00 .

11.7 Wash three times with PBS (pH 7.4).

11.8 To stain cellular actin, add 200 µL of PBS (pH 7.4) containing 0.15 µM Alexa Fluor 488-Phalloidin and incubate for 00:30:00 .

11.9 Wash three times with 500 µL PBS (pH 7.4).

Pause Point: If not imaging immediately, add sodium azide to the wells to a final concentration of 5 mM, wrap the sides of the plate with parafilm, and store at 4 °C . Plates can be stored for a minimum of 1 week.

Imaging & data analysis

1d

12 Imaging

12.1 Start the point-scanning or spinning disc microscope according to manufacturer's procedure.

12.2 Adjust the microscope parameters, including e.g. laser intensity, exposure time, camera EM gain, to obtain properly exposed fluorescence images in four channels: Cellular F-Actin (Phalloidin - Alexa Fluor 488), DAAM-particles (5/6-TAMRA cadaverine), exposed DAAM-particle surface (Secondary antibody - Alexa Fluor 647) using a 60 - 100x, high-NA (~1.4) oil objective. Specifically, ensure appropriate:

- **Z Range:** Capture images with a broad Z range, extending at least 5 µm above and 5 µm below the DAAM-particles.
- **Step Size:** Use a sufficiently fine z-step size (~ 0.15 µm)
- **Signal-to-noise ratio** (Fig. 4)

Note

Suggestion 9: Optimal imaging parameters are pivotal for obtaining high-resolution particle reconstructions and accurate force measurements. Avoiding pixel oversaturation, and achieving a good signal-to-noise ratio (SNR) are essential. SNR can be calculated as S/STD(background), where S is the mean foreground (particle) signal and STD the standard deviation of the background (particle surroundings). A SNR of around 100 is optimal[1]. Higher SNR does not lead enhanced edge localization precision but may increase acquisition times.

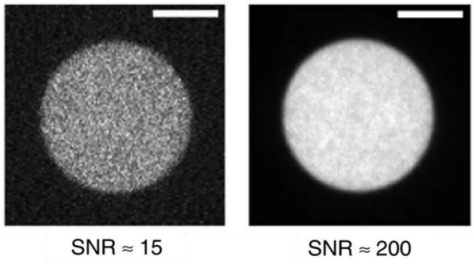

Figure 4: Confocal images of DAAM-particles. Suboptimal signal-to-noise ratio (SNR) (left) versus good SNR (right). Scale bar = 5 µm. Adapted from Vorselen et al., 2020.

12.3 Acquire images of 50 – 100 particles per imaging condition.

13

Note

Suggestion 10: The full image analysis requires MATLAB and Python, but doesn't require any prior experience and knowledge with programming.

3D particle reconstruction

Download the **MATLAB code** for analysing confocal images and deriving particle shape

13.1 Open Readme.pdf file and follow the instructions for installing the required Matlab software and packages.

13.2 Open "ImageAnalysis.m" in MATLAB, and add all the DAAMparticle_Shape_Analysis-master folders and subfolders to the Matlab file path. To do so, first click "Browse for folder" in the upper left corner above the current folder window, then, in the current folder window, rightclick on the DAAMparticles_Shape_Analysis folder (-> Add to Path -> Selected Folder and Subfolders).

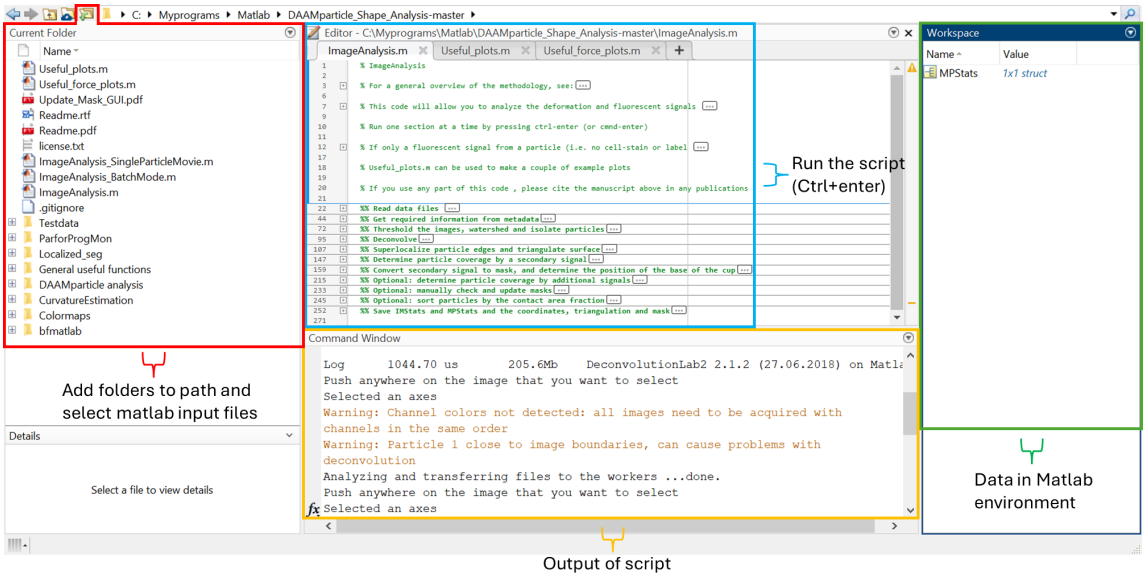

Figure 5: the matlab environment with corresponding sections.

13.3 For the next few steps (13.3 - 13.11), run the corresponding code sections by selecting a code section and pressing Ctrl+Enter. A testcase is added to reproduce the output images of the accompanying Plos One manuscript.

Run **Read data files**: Select your confocal image file in the window that opens. (Testcase: “ProtocolsIO_PlosOne_RAW_IgG_1.3kPa.tif” in the folder “Testdata”)

13.4 Run **Get required information from metadata.**
1. Select the DAAM-particle image in the window that opens (see Fig. 6).
2. Enter z-correction factor. Check Vorselen et al., 2020 how to estimate for your own data/microscope. (Testcase: z-correction factor = 1.1)
3. When prompted, enter pixel size values of the images. (Testcase: these values are “PixelSize x and y (µm)” = 0.109 and “Absolute PixelSize Z (µm)” = 0.2)

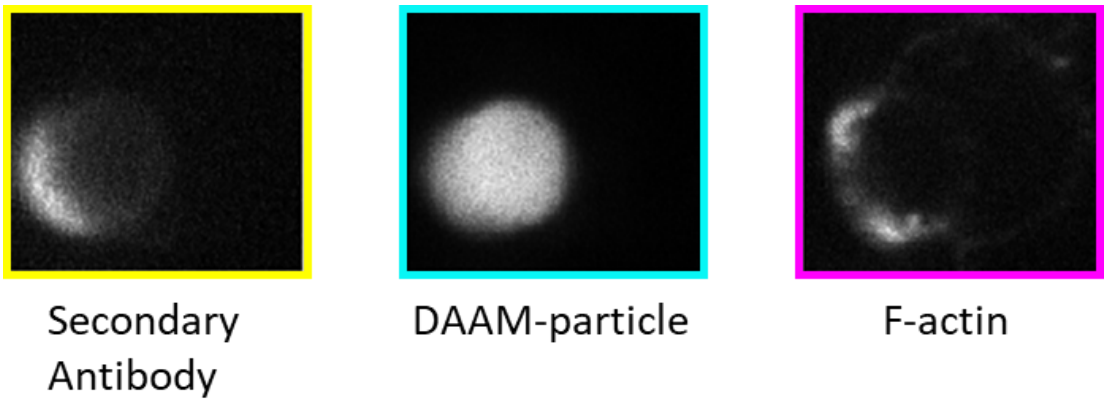

Figure 6: The prompted channels during the 3D particle reconstruction

Note

Troubleshooting 6: If not prompted, the pixel size data is directly taken from the metadata. It is advised to check if the pixel size values are correctly transferred. After running the code section “Get required information from metadata” check the pixel size values using IMStats(1).PixelSizeXY and IMStats(1).PixelSizeZ, and compare with stage micrometer measurements and chosen z step size. If incorrect, the pixelvalues can be modified using [IMStats.PixelSizeZ] = **value** and [IMStats.PixelSizeXY] = **value**.

13.5 Run **Threshold the images, watershed and isolate particles.** When prompted, use the default settings.

13.6 **Optional step.** Run **Deconvolve:** Only possible after measuring Point-Spread-Function (PSF) of your microscope. See supplementary Fig. 4 of Vorselen et al., 2020. Usually high-resolution can be achieved without deconvolution, but deconvolution can further improve resolution.

13.7 Run **Superparticle edges and triangulate surface.** When prompted, use default settings

13.8 Run **Determine particle coverage by a secondary signal** and select secondary antibody channel (Fig. 6)

13.9 **Optional step.** Run **Determine particle coverage by additional signals.** This can help make masks (see 13.10) more accurate in case of uneven antibody staining**,** and also allows inspecting of F-actin relative to the observed particle deformations. When prompted, select F-actin channel (Fig. 6). (Testcase: select the F-actin channel)

13.10 Run **Convert secondary signal to mask, and determine the position of the base of the cup.** When prompted, use default settings. This finalizes the analysis.

13.11 To visually inspect the data, open **Usefulplots.m** and run the following sections:

Plot triangulated surface of particles: This shows the particle 3D shape and a 2D projection of the particle surface (see accompanying Plos One manuscript, Fig. 2d). The particles can be rotated to be inspected from any angle in the matlab figure window.

Plot secondary stain on particle for both IMStain (F-actin) and _ch3 channels to make visualizations of these channels on the particle. The color can be changed at plot options, otherwise use the default options. (Testcase: Grey is actin signal, magenta is secondary antibody signal. See accompanying Plos One manuscript, Fig. 2d).

Matlab figures can be saved .tiff or .eps files for external viewing.

Note

Troubleshooting 7: Run **manually check and update masks** in case of uneven secondary antibody staining or other issues leading to masking inaccuraries.

13.12 To save output, run **Save the edge coordinates, triangulation and mask** in **ImageAnalysis.m** to create CSV files with the corresponding data. The IMStats and MPstats that are also saved can be opened in Matlab again to adapt parameters and (re)create figures.

Note

Suggestion 13: Additional data can be acquired from the 3D particle reconstructions, such as local curvatures, using the remaining sections. Moreover, data about the sphericity, contact area and fraction engulfed can be obtained in the MPStats.

14 Force Analysis

Download the **Python code** for deriving traction forces.

14.1 Create a new folder in ShElastic-master and put the following MATLAB output files in this folder. (Testcase: ShElastic-master -> testdata):

- MyFile_Connectivity (Testcase: Particle01_Connectivity)
- MyFile_Coordinates_Cart_smoothed (Testcase: Particle01_Coordinates_Cart_smoothed)
- MyFile_Mask_1um_dilated (Testcase: Particle01_Mask_1um_dilated)

MyFile should be replaced with the name corresponding to the output files of MATLAB.

14.2 Open the README.md and follow the suggested installation method to open the script:

- Install **Anaconda**
- Open Anaconda Navigator and import ShElastic.yml (located in the ShElastic folder) as **new environment**
- Activate the ShElastic environment
- Press home and open jupyter notebook
- Forward to the (ShElastic-master -> examples ->) "Case08-Hydrogel_Microparticles_as_Force_Sensors.ipynb"

14.3 Go to Input information and change the following parameters (Testcase: Parameters shown below (Fig. 7))

```
########################## change inputs here ##############################
# Data file name
datadir = '../testdata'
smoothed = 'smoothed'
dilated = '_1um_dilated'
#dilated = '_softedge'
shapename = 'Particle01'
datafile = os.path.join(datadir, shapename+'_Coordinates_Cart_'+smoothed+'.csv')
connfile = os.path.join(datadir, shapename+'_Connectivity.csv')
maskfile = os.path.join(datadir, shapename+'_Mask'+dilated+'.csv')
print('Cartesian coordinates: %s'%datafile)
print('Node connectivity: %s'%connfile)
print('Traction-free mask: %s'%maskfile)

# Material properties
mu0 = 1300/3; nu0 = 0.499;
```

Adapt to file location with the saved files from matlab

Adapt to the Myfile names

Adapt the mu0 value to the young's modulus (in Pa), (**leave the /3)**

Figure 7: Input data for deriving traction forces

14.4 Go to Kernel (top bar of the screen) and press: "Restart & Run All". This will run the entire code. The output file (output_data.mat) is located in the folder ShElastic (see above) in the subfolder examples.

> Note
>
> Depending on the device and complexity of the deformed shape, the code may take from minutes to several hours. It will save checkpoints during the analysis which makes it possible to perform the analysis over more runs (rerun the entire code).

14.5 To visualize and save figures of the analyzed forces, start MATLAB and open the file **Usefulforceplots.m** (located in DAAMparticle_Shape_Analysis-master folder). Import **output_data.mat** obtained during the Python analysis.

14.6 Run the code section **Plot forces in 2D** to visualize the forces on the particle (See accompanying Plos One manuscript, Fig. 2e). Matlab figures can be saved .tiff or .eps files for external viewing.

> Note
>
> **Suggestion 14:** You can change the arrow size and density by changing the parameters quiversize and reduce_dens in the code respectively.

Protocol references

1. Vorselen, D. et al. Microparticle traction force microscopy reveals subcellular force exertion patterns in immune cell–target interactions. Nature Communications 2020 11:1 11, 1–14 (2020).