CHAPTER

20 Generation of biocompatible droplets for in vivo and in vitro measurement of cell-generated mechanical stresses

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Abstract

Here we describe a detailed protocol to produce biocompatible droplets that permit the measurement of mechanical stresses at cell and tissue scales. The droplets can be used as force transducers in vivo, ex vivo, and in vitro, to measure mechanical stresses in situ, in three dimensions and time. Versatile and modular droplet coatings using biotinylated molecules, such as ligands for specific adhesion receptors, enable the targeting of specific tissues or cells. Droplet sizes can be varied to measure forces at different scales (tissue and cell scales) and the range of measurable mechanical stresses ranges within approximately 0.3−100 kPa. The protocol described in this chapter is divided in three sections. First, we describe the generation and stabilization of biocompatible droplets. Next, we explain the steps necessary to functionalize the droplet surface. Finally, we describe how to characterize the mechanical properties of the droplets, so that they can be used as calibrated mechanical probes. The procedure to generate, stabilize, and functionalize the droplets is straightforward and can be completed in about 3 h with basic laboratory resources. The calibration of the droplet's mechanical properties to perform quantitative stress measurements is also straightforward, but requires the proper equipment to measure interfacial tension (such as a tensiometer). Calibrated droplets can be used to quantify cell-generated mechanical stresses by analyzing the tridimensional shape of the droplet.

INTRODUCTION

There exist several techniques that allow the measurement of forces generated by cells. It is not our intention to provide here an exhaustive review of all existing techniques, but we briefly mention some of the methods that have been previously used to quantify cell-generated mechanical stresses in animal cells. One of the most important distinctions between the techniques to measure cellular forces is their ability to do so in vitro (i.e., in cells in culture) or in vivo within living tissues.

There are many techniques that permit the measurement of different aspects of cellular forces in cultured cells: (1) Traction force microscopy allows the measurement of cell traction forces for individual cells, cells within two-dimensional (2D) monolayers (see, e.g., Serra-Picamal et al. (chapter 17 of this volume), Martiel et al. (chapter 15 of this volume), and Gupta et al. (chapter 16 of this volume)) and even for cells embedded in three-dimensional (3D) synthetic gels (Dembo & Wang, 1999; Legant et al., 2010; Trepat et al., 2009). (2) Micropillar beds allow measurements of cell traction forces in 2D layers (Tan et al., 2003). (3) Fluorescence resonance energy transfer sensors allow molecular tension measurements inside cells or in the extracellular matrix on which they adhere and exert traction forces (see LaCroix et al. (chapter 10 of this volume), Grashoff et al. (2010), and Smith et al. (2007)). (4) Micropipette aspiration has been used to measure the adhesion strength between cells (see Biro and Maitre (chapter 14 of this volume) and Maitre et al. (2012)) and it also allows the measurement of both the interfacial tension and mechanical properties of cellular aggregates (Guevorkian, Gonzalez-Rodriguez, Carlier, Dufour, & Brochard-Wyart, 2011). (5) More recently, a tensile testing-based

technique has been developed to measure mechanical properties of epithelial monolayers (Harris et al., 2012).

The availability of techniques allowing the measurement of cell-generated mechanical stresses in vivo, however, is still very limited. While laser ablation has been used to probe the mechanics of living tissues (Hutson et al., 2003; Rauzi & Lenne, 2011), the interpretation of the results obtained is quite complex. This technique allows precise perturbations of the mechanics of living tissues, however, quantitative measurements of cellular forces or mechanical stresses cannot be performed because the mechanical properties of the tissue surrounding the ablation site are generally unknown. Despite not being a measurement technique per se, force inference methods can, under some assumptions that may be more or less restrictive depending on the tissue of interest, infer the cortical tensions and cell pressures throughout the tissue from the cell shapes (see, e.g., Veldhuis et al. (chapter 18 of this volume) and Brodland et al. (2014)). Finally, a new technique, which complements laser ablation and inference methods, uses oil droplets as in vivo force transducers and allows quantitative measurements of cell-generated stresses in living embryonic tissues in 3D and time (Campàs et al., 2014).

Here, we describe how to generate and characterize biocompatible droplets that can be used as force transducers in vivo, ex vivo, or in vitro (Campàs et al., 2014). Once produced and calibrated, these functionalized oil droplets are injected in between the cells forming a living tissue or placed between cells in culture. Specific coating of the droplet surface allows the cells to push and pull on the droplet via specific molecular adhesions, which induces droplet deformations that provide a direct readout of physiologically relevant cellular stresses. It is thus possible to quantify cellular stresses (both compressions and tensions surrounding the droplet) in 3D and time by knowing the precise shape of the droplet in 3D and its temporal shape changes. This can be done using confocal microscopy, or any other technique allowing high-resolution fluorescence imaging in 3D. When droplets are fully embedded in tissues, only anisotropic stresses can be measured. However, in cases where the droplet is only partially embedded, such as in epithelial tissues or cells in culture, this technique allows for the full quantification of stresses (Campàs et al., 2014).

The ability to modify the surface chemistry of the droplet in a modular way (through the use of different surfactants or ligands; see below) and the control over the droplet interfacial tension, which allows measurement of a broad range of cellular stresses (0.3−100 kPa), make this a very flexible technique that can be adapted to many different experimental conditions.

1. METHODS

1.1 GENERATION AND STABILIZATION OF BIOCOMPATIBLE DROPLETS

The generation of stable, biocompatible droplets can be achieved with different methods. Polydisperse emulsions, containing droplets of variable sizes, can be easily

generated by bulk emulsification, whereas monodisperse emulsions can be obtained using standard droplet microfluidic techniques (Baroud, Gallaire, & Dangla, 2010; Garstecki, Fuerstman, Stone, & Whitesides, 2006; Seemann, Brinkmann, Pfohl, & Herminghaus, 2012; Teh, Lin, Hung, & Lee, 2008). In this protocol, we only describe how to produce polydisperse emulsions with droplet sizes within an approximate range of 3−80 μm in diameter. The protocol can be easily modified to achieve a different range of droplet sizes by filtering droplets with filters of different pore sizes. Importantly, droplets smaller than cell size are typically internalized by cells, preventing intercellular force measurements. On the other hand, if used to measure cellular stresses in vivo, very large droplets may considerably affect the proper development of the tissue. Thus, droplets should be selected to approximately match the average size of the cells being analyzed.

1.1.1 Materials

- MilliQ Deionized (DI) water (all experiments should use filtered DI water).
- Fluorinert FC-70 Electronic Liquid (3M, ref: AM000000290).
- DSPE-PEG(2000)-biotin (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl(polyethylene glycol)-2000); Avanti Polar Lipids, Inc., ref: 880,129P).
- Bovine serum albumin (BSA) (Sigma−Aldrich, ref: A7888).
- 4 mL glass vial (VWR, ref: 89,210-120).
- 0.2 μm syringe filter (Pall Corporation, ref: AP-4799).

1.1.2 Equipment

- Sonicator (e.g., Branson Ultrasonics, ref: 1510-DTH).

1.1.3 Method

Here we describe in detail the method used to generate a stable bulk emulsion of droplets that can be used for the measurement of cell-generated mechanical stresses. Several types of oils can be used to generate the droplets, but only specific oils have the required properties to measure cellular forces. Vegetable oils can be used in applications that do not involve cells or tissues (e.g., in vitro assays with reconstituted proteins, cell extracts, etc. (Boukellal, Campás, Joanny, Prost, & Sykes, 2004; Trichet, Campàs, Sykes, & Plastino, 2007)), as these oils mix easily with cell membranes. Fluorocarbon oils have the appropriate physical and chemical properties for experiments with cells and tissues, namely: (1) they are immiscible in hydrocarbon oils, preventing undesired mixing with cell membranes (Köster et al., 2008; Krafft & Riess, 2009; Riess, 2009); (2) they have been extensively used in biological studies and repeatedly shown to be biocompatible (Keese & Giaever, 1983; Marchbank, 1995; Riess & Krafft, 1998); (3) their high density ($\sim$1500−2000 kg/m^3) substantially simplifies the preparation and coating of droplets. Finally, other oils could potentially be used (silicon oils, mineral oils, etc.) but have not yet been tested specifically for the application of cellular force measurements.

In order to stabilize the emulsion and prevent droplets from coalescing, several types of surfactants can be used. Fluorocarbon emulsions used in medical

applications (e.g., as blood substitutes (Riess, 2001)) have been previously stabilized using phospholipids (Bertilla, Thomas, Marie, & Krafft, 2004; Riess, 2001). We use DSPE, a phospholipid, coupled to a poly-(ethylene glycol) spacer linked to biotin (DSPE-PEG(2000)-biotin), as surfactant in our experiments. The PEG polymer prevents nonspecific interactions between cells and droplets, whereas the biotin group enables further functionalization of the droplets (see below). Similar molecules have previously been used to functionalize lipid vesicles (Leduc et al., 2004) as well as stabilize and functionalize oil-in-water emulsions (Fattaccioli, Baudry, Henry, Brochard-Wyart, & Bibette, 2008; Hadorn et al., 2012; Jorjadze, Pontani, Newhall, & Brujic, 2011; Pontani, Jorjadze, Viasnoff, & Brujic, 2012). Importantly, using a phospholipid along with a (semifluorinated alkane) cosurfactant inside the oil droplet can stabilize and vary the interfacial tension of fluorocarbon-in-water emulsions (Bertilla et al., 2004; Campàs et al., 2014; Krafft & Riess, 2009).

To obtain a stable bulk emulsion of fluorocarbon droplets in water follow these steps:

1. Prepare 15 mL of a 1 mg/mL (or 0.1% w/w) BSA solution in water. Filter the solution with a 0.2 μm filter. Any surface (glass vials, pipette tips, etc.) the droplets will be in contact with should be coated with BSA to prevent the possibility of the oil droplets wetting the surface.
2. Filter the FC-70 oil with a 0.2 μm filter.
3. Pour approximately 1 mL of the BSA solution into the 4 mL glass vial to coat it with BSA. This can be done in many ways (e.g., simply rotating the vial), as long as the BSA fully coats the vial. The excess BSA in the coated vial can be reused to coat other surfaces during the experiment.
4. Rinse the BSA-coated vial with DI water.
5. Add 1 mL of DSPE-PEG(2000)-biotin surfactants in aqueous solution at a final concentration of 0.2 mM to the glass vial. (It is convenient to have a stock solution of DSPE-PEG(2000)-biotin in water. The critical micelle concentration of these lipids is approximately 1 μM (Ashok, Arleth, Hjelm, Rubinstein, & Onyüksel, 2004)).
6. Mix gently and sonicate the solution for 1 min.
7. Coat a 1 mL pipette tip with BSA by aspirating and dispensing the 0.1% BSA solution. This tip will be used to shear the droplets.
8. Add 150 μL of filtered FC-70 oil to the 0.2 mM phospholipid solution in the glass vial.
9. Cap the vial and vigorously shake it to create a coarse emulsion (Figure 1(A)).
10. Use the BSA-coated 1 mL pipette tip to aspirate and dispense the mixture in order to shear the emulsion and generate smaller droplets. To do so, aspirate the mixture, position the pipette tip against the bottom of the glass vial, and then strongly dispense the mixture. The pipette tip should be in contact with the glass vial, so that droplets are strongly sheared when flowing out of the tip (Figure 1(B)).

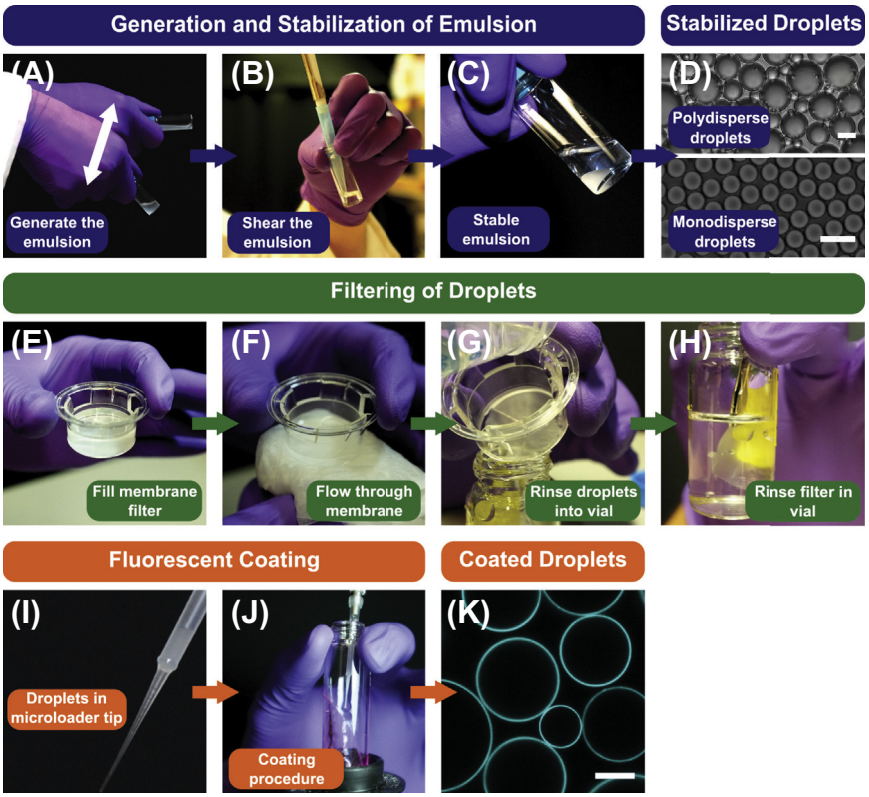

FIGURE 1

Production and functionalization of droplets. (A) Vigorous shaking to generate a bulk emulsion. (B) A pipettor is used to create a fine emulsion from the coarse emulsion generated in A. Aspirate the droplets and then dispense quickly and energetically with the pipette tip in contact with the glass vial. This process generates large shear stresses at the pipette tip that will create a fine emulsion. (C) Image of stable droplets in the vial after generation. If large droplets are visible in the vial then the previous step should be repeated, until a smooth emulsion is generated. (D) Polydisperse and monodisperse droplets, created by bulk emulsification and microfluidics, respectively. (E) Filtering droplets by size. Image of the membrane filter containing droplets in DI water. (F) Filter droplets smaller than the 3 μm pore size of the membrane by inducing a flow through it with a Kimwipe. (G) An edge of the filter is cut, and abundant DI water is used to rinse the droplets into another vial. (H) The cut filter is submerged in the vial using tweezers. This helps to recover any droplets that may remain on the filter. (I) Droplets loaded into the microloader pipette tip prior to coating. The tip may be cut to ease handling of the droplets—the inner diameter at the end of the tip is approximately 140 μm. However, creating a larger orifice will increase the probability of simultaneous dispensing of droplets, which should be avoided, as it may lead to droplets adhering to each other. (J) The fluorescent streptavidin solution is vortexed while droplets are dispensed using a microloader pipette tip. (K) Droplets coated with fluorescent streptavidin from a stable, polydisperse emulsion. Scale bars, 25 μm.

11. Let the droplets settle for 5 min and repeat this shearing process (typically three times) until the emulsion appears very smooth and no large droplets are visible. Since the oil has a density that is approximately twice that of water, the droplets will collect at the bottom of the vial (Figure 1(C)).
12. The vial containing the emulsion can be stored at 4 °C or room temperature.

This procedure generates a polydisperse (or monodisperse, should microfluidics be used) fluorocarbon emulsion stabilized by DSPE-PEG(2000)-biotin surfactants. Figure 1(D) shows emulsions generated by bulk emulsification (method described above) and microfluidics. The emulsion will be stable for weeks (both at 4 °C and room temperature) due to the excess of surfactants in solution; however, some degree of coarsening occurs and emulsions stored for a year typically contain droplets larger than the original ones. Moreover, the phospholipids may lose functionality over long periods of time. We recommend using the prepared droplets within 1 month of their preparation.

1.2 FUNCTIONALIZATION OF DROPLETS

Here we describe how to fluorescently label and functionalize the droplets produced in the previous section.

1.2.1 Materials

- MilliQ DI water.
- Fluorescent streptavidin conjugate (e.g., Alexa Fluor fluorescent streptavidin conjugates; Life Technologies).
- Biotinylated adhesion molecule. The choice of adhesion molecule depends on the application (e.g., ligands with an exposed arginine−glycine−aspartate (RGD) sequence for adhesion to integrin receptors).
- BSA (Sigma−Aldrich, ref: A7888).
- Microloader 20 μL pipette tip (Eppendorf, ref: 930,001,007).
- 11 mL glass vial (VWR, ref: 66,009-559).
- 20 mL glass vial (VWR, ref: 93,001-640).
- 3 μm membrane filter (Sigma−Aldrich, ref: CLS3414).
- Aluminum foil (Sigma−Aldrich, ref: 266,574).
- Disposable scalpel (Fisher Scientific, ref: 02-688-78).

1.2.2 Equipment

- Vortex mixer (e.g., Scientific Industries, Inc., ref: SI-0236).

1.2.3 Method

The droplets are first coated with fluorescent streptavidin conjugates. At the end of this initial coating, the droplets' surface will be saturated with streptavidin molecules (enabling further functionalization via biotinylated molecules) and visible under fluorescence microscopy. The choice of the fluorophore depends on the application, but a wide range of fluorescent streptavidin conjugates are commercially available.

During this initial coating, it is important to prevent as much as possible the contact between droplets; indeed, during this coating procedure, if droplets partially coated with streptavidin (with biotin groups at their surface not yet coupled to streptavidin) come into contact, they can bind to each other. This issue is addressed in the protocol below by adding droplets into a streptavidin solution through a very fine capillary, so that droplets reach the solution almost one at a time. While this method generates droplets nicely coated with streptavidin, a small fraction of droplets adhere to each other. Although it is not explained within this protocol, microfluidics could be used to optimize the results (Teh et al., 2008).

In a second coating, the selected biotinylated ligands for cell adhesion are bound to the streptavidin molecules on the droplet surface. These ligands will bind specific adhesion receptors at the cell surface, enabling the cell to pull on the droplets in addition to pushing them.

We now detail the specific protocol to perform both coatings:

1. Place approximately 2 mL of the 0.1% BSA solution onto a 3 μm membrane filter (Figure 1(E)).
2. Pull the BSA solution through the filter by placing a Kimwipe underneath, inducing capillary flow (Figure 1(F)).
3. Rinse the filter with DI water by pouring approximately 2 mL into the filter and pulling it through the membrane generating a capillary flow with a Kimwipe. Repeat the rinsing twice.
4. Add 1 mL of DI water in the filter.
5. With a BSA-coated pipette tip, take the previously stabilized emulsion (see previous section) and place it onto the filter (keeping the tip submerged in the DI water).
6. Use a Kimwipe to induce a capillary flow through the filter, thereby removing small droplets. Keep adding water so that the droplets on the filter are always submerged. After this step, droplets with a diameter larger than the pore size (3 μm in this case) should remain on the filter.
7. Coat a 20 mL vial with BSA, rinse it with DI water, and fill it approximately one-third of the way full with DI water.
8. Place the filter containing the droplets over the vial and cut a small lip into it using a scalpel (Figure 1(G)).
9. Pour DI water on the top of the filter so that the droplets that remained on the filter flow into the vial (Figure 1(G)).
10. Next, cut the remaining perimeter of the membrane filter. Grab it with tweezers and dip it into the vial, moving the tweezers back and forth so that any remaining droplets may come off the filter (Figure 1(H)). Let the droplets settle for approximately 20 min. Then tilt the vial, so that the droplets accumulate on the lower edge (approximately 5−10 min); the tilting of the vial helps to collect and rinse the droplets.
11. Carefully, with the droplets stored in the tilted vial, remove the majority of the liquid in the vial, leaving just enough water to cover the emulsion. It is

important to do this carefully so that the droplets do not disperse in solution. In case they disperse, let the droplets settle to the edge of the vial again by keeping the vial titled and undisturbed for approximately 10 min.

12. Replenish the vial with DI water carefully without disturbing the droplets. The goal is to thoroughly rinse the droplets with DI water. Adding water too quickly will cause the droplets to disperse in the bulk, and they will need to settle again before continuing the rinsing steps.
13. Repeat this rinsing procedure three times, always fully filling the vial with DI water and then removing it as indicated above. After this dilution step, store the glass vial tilted, so that the emulsion can be transferred easily.
14. Prepare an aqueous solution of the fluorescent streptavidin molecules in a BSA-coated, 11 mL glass vial. Typically, a working concentration of 2 μM is used to ensure the saturation of the droplet surface with fluorescent streptavidin molecules.
15. Coat a microloader pipette tip with BSA by aspirating and dispensing the solution. The tip may be cut to ease handling of the droplets—the inner diameter at the end of the tip is approximately 140 μm. However, creating a larger orifice will increase the probability of simultaneous dispensing of droplets, which should be avoided, as it may lead to droplets adhering to each other.
16. Aspirate some of the droplets into the microloader tip (Figure 1(I)).
17. Set the power of the vortex mixer between the 4–6 settings.
18. Grip the vial of streptavidin solution around the neck and tilt it slightly backward while pressing it down on the vortex mixer (Figure 1(J)). The solution should now be spiraling around the vial. Adjust the power until the streptavidin solution safely spirals around the vial without spilling.
19. Carefully lower the microloader tip containing the droplets into the rotating solution. The flow should break slightly around this point, if the tip is inside the streptavidin solution (Figure 1(J)).
20. Dispense the droplets slowly into the streptavidin solution. Once all droplets are dispensed, continue to vortex the solution for an additional 10 s to ensure they are fully coated. The droplets should now be uniformly coated with fluorescent streptavidin (Figure 1(K)).
21. Cover the vial with aluminum foil.
22. The streptavidin solution used to coat the droplets can be recovered by simply transferring it to another BSA-coated vial once the droplets have settled at the bottom of the vial. To do so, store the fluorescently coated droplets tilted, as described before, and wait approximately 30 min.
23. Without disturbing the droplets, transfer the streptavidin solution to a new glass vial and cover it with aluminum foil. Leave approximately 1 mL of the solution in the vial with the droplets.
24. Coat an 11 mL glass vial with BSA, rinse with DI water, and add 1 mL of DI water to it.
25. Coat a 1 mL pipette tip with BSA and transfer the coated droplets to this 1 mL of DI water.

26. Pipette the droplets up/down. Let them settle for 20 min.
27. Rinse the droplets as specified in the previous section, namely by filling the vial and subsequently removing the solution without disturbing the droplets. Repeat the rinsing procedure three times. After this step, the emulsion should be visibly colored due to the fluorescent molecules at their surface.

The droplets resulting from this procedure are coated with fluorescent streptavidin and can either be stored (preferably at 4 °C) or be further coated with a biotinylated adhesion molecule of choice.

To perform the second coating, with the biotinylated molecules of choice, follow the next steps:

1. Prepare a glass vial (of 11 mL capacity), coated with BSA, containing 500 μL of the biotinylated adhesion molecules solution at the desired concentration for coating purposes. The solution can be in either water or buffer, depending on type of biotinylated molecule used in this second coating. Biotinylated mouse E-cadherin antibodies (R&D Systems) have been previously used to measure stresses in mammary epithelial cell aggregates (Campàs et al., 2014). For measurements in tooth mesenchymal cell aggregates and the dental mesenchyme of mouse mandibles, RGD peptides *cyclo*-[Arg-Gly-Asp-d-Phe-Lys(Biotin-PEG-PEG)] (Peptides International) have been previously used (Campàs et al., 2014).
2. Aspirate some of the previously prepared droplets and dispense them into the solution containing the biotinylated ligands.
3. Aspirate and gently dispense the droplets in the solution with a pipettor.
4. Prepare a 20 mL vial, coated with BSA, and add approximately 5 mL of DI water. Coat a pipette tip with BSA and transfer the droplets into this vial. Let them settle for about 15 min.
5. Rinse the droplets as specified in the previous section, namely by filling the vial and subsequently removing the solution without disturbing the droplets. Repeat the rinsing procedure three times.

At this point of the protocol, stable and functionalized droplets have been obtained that can be used as force transducers to measure cell-generated mechanical stresses. While the emulsion of functionalized droplets is quite stable for weeks to months, the molecules used to coat the droplets may degrade, limiting their usable lifetime. Hence, they should be used as soon after preparation as possible, preferably within 1 week.

1.3 CHARACTERIZING THE MECHANICAL PROPERTIES OF THE DROPLETS

In order for the droplets to act as calibrated probes, their interfacial tension needs to be determined. There are many different methods to measure the interfacial tension between two fluids (Du Noüy ring, Wilhelmy plate, pendant drop methods, rotating

droplets, etc.). We use the pendant drop method because it allows precise measurements of interfacial tension with small volumes of reagents (this is important when only a limited amount of surfactants is available). We explain below in detail how to measure the interfacial tension at each step of the droplet coating procedure described in the previous section, and also, the interfacial tension of the coated droplet in contact with the fluid that will be used during the force measurement experiments with cells.

1.3.1 Materials

- MilliQ DI water.
- Fluorinert FC-70 Electronic Liquid (3M, ref: AM000000290).
- DSPE-PEG(2000)-biotin (Avanti Polar Lipids, Inc., ref: 880,129P).
- Fluorescent streptavidin conjugate (e.g., Alexa Fluor fluorescent streptavidin conjugates. See Life Technologies).
- Solution to be used during force measurements with cells/tissues. This solution depends on the application. For example, if droplets were used to measure cellular stresses between cells in culture, this would be the cell culture media used (e.g., Dulbeccos Modified Eagles Medium supplemented with fetal bovine serum—the precise composition of cell culture media will depend on the cells used in the experiments).
- 1 mL plastic syringe (Air Tite Products Co, ref: A1).
- 18 gauge syringe needle (Kimble Chase Life Science and Research Products, ref: 868,280-1801).
- Saint-Gobain Tygon Sanitary Silicone Tubing, I.D. 1/32″ (Fisher Scientific, ref: 02-587-1A).
- 5-min epoxy (ITW Devcon, ref: 14,250).
- Glass slide (VWR, ref: 16,004-368).
- Plastic cuvette (Sigma−Aldrich, ref: Z637157).

1.3.2 Equipment

- Theta (Biolin Scientific) optical tensiometer (Figure 2(A)) or a homemade optical tensiometer. As mentioned above, other methods can be used if a pendant drop tensiometer is not available.
- Bunsen burner (VWR, ref: 89,038-530).
- Sonicator (e.g., Branson Ultrasonics, ref: 1510-DTH).

1.3.3 Method

The procedure described above for the functionalization of the droplets has several coating steps and it is informative to know the interfacial tension changes at each step. To be able to measure interfacial tension while exchanging solutions, a homemade perfusion chamber is used (see below). This allows continuous measurements of interfacial tension during the successive changes of the solutions involved in the droplet coating procedure.

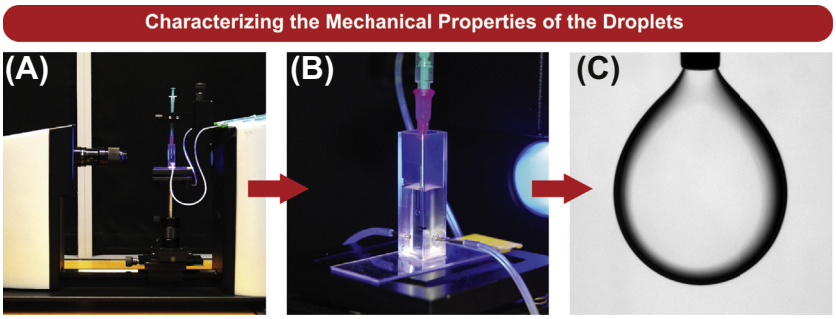

FIGURE 2

Measurement of the interfacial tension using the pendant drop method. (A) Theta optical tensiometer setup. A homemade setup may also be used for experiments. (B) Perfusion chamber for continuous interfacial tension measurements during successive droplet coatings. (C) A pendant drop of FC-70 oil in water. The image settings should be optimized to focus the drop and give high contrast of its surface.

1. A homemade perfusion chamber (Figure 2(B)) can be made as follows:
   - **a.** Cleave 2 of the 18 gauge syringe needles from their luer lock fittings.
   - **b.** Heat the needle's tip over a Bunsen burner, and then bring them in contact with a cuvette, pushing through to create the inlet/outlet.
   - **c.** Mix the 5-min epoxy base and curing agent. Apply the epoxy to the outside of the cleaved needles to secure them and seal the inlet/outlet.
   - **d.** The 1/32″ I.D. tubing can be attached to the inlet/outlet needles to enable successive changes of the solutions for coating the pendant drop (Figure 2(B)).
   - **e.** Using the epoxy, the bottom of the cuvette may be adhered to a glass slide to keep it stable during measurements.
2. Prepare a 1 mL syringe and needle containing FC-70 oil. The needle gauge is important: a large gauge needle (thus a small diameter) can provide false values because the hanging drop does not have enough weight to deform itself. On the other hand, smaller gauge needles (thus a larger diameter) cannot be used to measure low interfacial tensions because the droplets will fall due to the larger volume needed. It is important to test different needle gauges to find the optimal range of gauges that allow reliable measurements.
3. Immerse the needle into DI water in the perfusion chamber and create a droplet until it is hanging from the needle tip (Figure 2(B)).
4. Measure the interfacial tension of the FC-70 droplet in DI water. The imaging settings of the droplet should be optimized to be in focus and give high contrast (Figure 2(C)).
5. Sonicate the aqueous DSPE-PEG(2000)-biotin stock solution for 1 min.
6. Introduce a 0.2 mM solution of DSPE-PEG(2000)-biotin into the perfusion chamber though the inlet.

7. Carefully mix the contents in the chamber. Mixing too vigorously may cause the droplet to fall and the experiment would need to be started again.
8. Measure the interfacial tension of the FC-70 droplet in the lipid solution as a function of time until it reaches a constant value.
9. In order to rinse the chamber, add DI water into the perfusion chamber through the inlet and remove it through the outlet. Ensure that the droplet is always immersed in the fluid while rinsing. Assuming the droplet is immersed in approximately 500 μL of solution to start, adding and subsequently removing 50 mL of DI water should be enough for rinsing purposes.
10. Measure the interfacial tension.
11. Remove as much DI water as possible while still leaving the droplet submerged.
12. Introduce the streptavidin solution (we use a working concentration of 2 μM) into the chamber.
13. Measure the interfacial tension as a function of time until it reaches a constant value.
14. Rinse the chamber with DI water as previously explained.
15. Again, remove as much DI water from the chamber as possible, keeping the droplet submerged.
16. Introduce the solution containing the biotinylated adhesion molecules of choice into the chamber.
17. Rinse the chamber with DI water as previously explained.
18. Measure the interfacial tension as a function of time until it reaches a constant value.
19. Again, remove as much DI water from the chamber as possible, keeping the droplet submerged.
20. Introduce the solution where the force measurements will be performed into the perfusion chamber (e.g., cell culture media if the droplets will be used to measure forces in cultured cells).
21. Measure the interfacial tension as a function of time until it reaches a constant value.
22. Finally, measure the interfacial tension at the temperature where the experiments will be performed. For instance, if the droplets will be used to measure cellular forces with cultured cells at 37 °C, then warm up the cell culture media to be used, add it into the perfusion chamber and measure the interfacial tension. If possible monitor the temperature during the measurement.

Once the interfacial tension is known, the oil microdroplets are functionalized and calibrated probes, meaning that the mechanical stresses generated by cells on the droplets can be quantitatively measured by analyzing the shape of the droplet. The relation between the droplet shape and the stresses applied by cells, as well as a description of the algorithms used to analyze the droplet shape in 3D, can be found in Campàs et al. (2014).

1.4 USE OF DROPLETS IN DIFFERENT APPLICATIONS

Fluorocarbon oil droplets can be used as cellular force transducers in vitro and in vivo. Below we discuss how the droplets can be used to measure cell-generated mechanical stresses in different applications.

1.4.1 In vitro

Droplets can be used in different in vitro situations to measure the mechanical stresses generated by individual cells, cells within cell monolayers, or cells in 3D aggregates in culture conditions.

1.4.1.1 Single cells and cells in 2D monolayers

To perform measurements with single cells, a droplet needs to be brought into contact with a cell. Once the cell adheres to the droplet, force measurements can be performed by simply imaging the system. The droplet can be precisely put in contact with cells using a microinjector system to dispense the droplet accurately. Alternatively, simply cover cultured cells with droplets and rinse the excess droplets after a few minutes, once the droplets adhere to the cells.

To measure mechanical stresses in cells forming a monolayer, the droplets need to be embedded within the monolayer. Starting with a low density of cells on the dish, disperse some droplets in between the cells. Upon growth, cells will contact the droplets and spontaneously embed them in the monolayer. If the cell adhesion strength between cells is very different than between cells and droplets, then the droplets will either be expelled from the monolayer or the cells will grow as monolayers on the droplet surface. Thus, the droplet size and surface coatings need to be optimized for each cell type to be studied, but reasonable initial estimates for the droplet size range and interfacial tensions in these experiments are 20−40 μm and 10−20 mN/m, respectively. In the case of single cells, oil droplets allow the direct quantification of the normal stresses applied by the cell on the droplet.

1.4.1.2 Cell aggregates

To prepare cell aggregates containing functionalized droplets in between the cells, suspend the cells at high density and add a desired quantity of functionalized droplets to the cells in suspension. Carefully mix the droplets and the cells. Centrifuge the mixture to obtain pellets of cells and droplets that can be cultured into compact cell aggregates over a period of several days (Campàs et al., 2014).

1.4.2 Ex vivo and in vivo

In order to perform force measurements in living embryonic tissue, whether ex vivo or in vivo, it is necessary to microinject the droplets in the tissue of interest. This requires a microinjection setup (such as the standard microinjections systems used in developmental biology) that we do not describe here. We assume that microinjectors are available to the user.

To measure forces ex vivo, first dissect the tissue of interest. Load a microneedle with a previously prepared emulsion of functionalized droplets (backloading the

needle works well; if droplets stick to the needle, coat the microneedle with BSA). Inject droplets using a microinjector setup immediately after the tissue dissection. Culture the dissected tissue containing the droplets until the damage caused by the injection is repaired and the droplet is embedded in the tissue (this may take as little as 30 min or 3−5 h depending on the tissue).

To inject the droplets in living embryos, simply load a microneedle with a previously prepared emulsion of functionalized droplets, and inject the droplets in the tissue of interest. Droplets can be injected one at a time or in small groups. Wait until the damage caused by the injection is repaired and the droplet is embedded in the tissue/embryo before starting the measurement of droplet deformations.

In the case of cell aggregates and in living tissue, if the droplets are fully embedded within the aggregate or tissue, this technique allows a quantitative measurement of the local anisotropic stresses. However, the local tissue pressure cannot be measured if the droplets are fully surrounded by cells. In case the droplet has a surface free of cells, then it is possible to measure both the local tissue pressure as well as the anisotropic stresses (Campàs et al., 2014).

2. DISCUSSION

The protocol described above allows the generation of biocompatible functionalized droplets to measure cell-generated mechanical stresses in vitro and in vivo. These droplets can be generated easily, with equipment available in most laboratories. Several droplet parameters can be modified to adapt the protocol to different applications and for different cell types. Here we explain how to vary or control some of them:

- **Surfactants for stabilization**. Many different surfactants can be used to stabilize the droplets. In the protocol described above we used DSPE-PEG(2000)-biotin for stabilization because it is a commercially available phospholipid with a biotinylated PEG molecule linked to the headgroup. However, other phospholipids or engineered block copolymers with hydrophilic/hydrophobic moieties can also be used. In some cases, if the cellular forces are strong enough, the surfactants may be pulled off from the surface of the droplet. Thus, it is important to choose the appropriate surfactants and ensure a high enough surfactant density on the droplet surface.
- **Interfacial tension**. The addition of a cosurfactant in the fluorocarbon phase can lower the interfacial tension of the fluorocarbon/aqueous interface in a concentration-dependent manner (Bertilla et al., 2004). The use of cosurfactants allows control over the range of mechanical stresses that can be measured. Lower interfacial tensions permit measurements of low stresses whereas large interfacial tensions should be used to measure high stresses. While many cosurfactants exist that can potentially be used, small fluorocarbon−hydrocarbon diblocks have been shown to considerably lower the surface tension when

combined with phospholipids (Bertilla et al., 2004; Krafft & Riess, 2009). High molecular weight fluorocarbon–hydrocarbon diblocks have been used to lower the interfacial tension of the droplets, which permits measurement of cellular stresses in vivo (Campàs et al., 2014). The interfacial tension can also be varied using combinations of different surfactants (phospholipids or fluorinated surfactants), or by changing the fluorocarbon oil (several fluorocarbon oils with different physicochemical properties are commercially available).

- **Droplet size**. Cellular force measurements require droplets of sizes on the cellular scale, whereas tissue-scale stress measurements can be done with larger droplets. The protocol described here only allows the generation of polydisperse emulsions with droplets sizes varying within a large range. In order to narrow down this range, polydisperse emulsions can be filtered to eliminate droplets above and below specified sizes (as described above and by Campàs et al. (2014)). However, if precise control of droplet size is desired, we recommend the use of droplet microfluidics. Alternatively, other techniques, such as the Couette cell apparatus (Mason & Bibette, 1996), allow the generation of monodisperse emulsions.
- **Droplet functionalization**. The ability to control the type and concentration of the ligands for cell adhesion receptors at the droplet surface allows the droplets to be used to measure cell-generated forces in many conditions. Importantly, molecules other than adhesion molecules (or in addition to adhesion molecules) can also be used to coat the droplets. In order to use them within the protocol described here, these molecules need to be biotinylated and added to the second coating step described above. While shear stresses on the droplet's surface cannot be measured with the version of the technique explained above, it might be possible to overcome this limitation in the future by coating the droplets with cross-linkable surfactants.

CONCLUSION

Successful measurement of cell-generated mechanical stresses in many different conditions can be achieved by adapting the functionalized oil droplets described in this protocol to a particular application. The use of droplets as force transducers can help elucidate how mechanics affects essential cellular behavior, such as cell proliferation, migration, and even differentiation, within living embryonic tissues. Moreover, it can also be used in cell aggregates and other in vitro systems that allow a precise control of environmental conditions to quantify intercellular forces. Finally, droplet-based force transducers may also help in tissue engineering and regenerative-based medical applications, as a clear understanding of the mechanics of living tissues can guide the development of new materials and cell scaffolds with specific mechanical properties to mimic the native tissue. Overall, the versatility of droplet-based force transducers can open new avenues to study the mechanics of living tissues and cellular assemblies, as well as its control at the molecular level.

REFERENCES

Ashok, B., Arleth, L., Hjelm, R. P., Rubinstein, I., & Onyüksel, H. (October 2004). In vitro characterization of PEGylated phospholipid micelles for improved drug solubilization: effects of PEG chain length and PC incorporation. *Journal of Pharmaceutical Sciences, 93*(10), 2476−2487.

Baroud, C. N., Gallaire, F., & Dangla, R. (August 2010). Dynamics of microfluidic droplets. *Lab on a Chip, 10*(16), 2032−2045.

Bertilla, S. M., Thomas, J. L., Marie, P., & Krafft, M. P. (May 2004). Cosurfactant effect of a semifluorinated alkane at a fluorocarbon/water interface: impact on the stabilization of fluorocarbon-in-water emulsions. *Langmuir, 20*(10), 3920−3924.

Boukellal, H., Campás, O., Joanny, J.-F., Prost, J., & Sykes, C. (June 2004). Soft *Listeria*: actin-based propulsion of liquid drops. *Physical Review E, 69*(6), 061906.

Brodland, G. W., Veldhuis, J. H., Kim, S., Perrone, M., Mashburn, D., & Hutson, M. S. (January 2014). CellFIT: a cellular force-inference toolkit using curvilinear cell boundaries. *PLoS One, 9*(6), e99116.

Campàs, O., Mammoto, T., Hasso, S., Sperling, R. A., O'Connell, D., Bischof, A. G., et al. (February 2014). Quantifying cell-generated mechanical forces within living embryonic tissues. *Nature Methods, 11*(2), 183−189.

Dembo, M., & Wang, Yu-L. (April 1999). Stresses at the cell-to-substrate interface during locomotion of fibroblasts. *Biophysical Journal, 76*(4), 2307−2316.

Fattaccioli, J., Baudry, J., Henry, N., Brochard-Wyart, F., & Bibette, J. (2008). Specific wetting probed with biomimetic emulsion droplets. *Soft Matter, 4*(12), 2434−2440.

Garstecki, P., Fuerstman, M. J., Stone, H. A., & Whitesides, G. M. (March 2006). Formation of droplets and bubbles in a microfluidic T-junction-scaling and mechanism of break-up. *Lab on a Chip, 6*(3), 437−446.

Grashoff, C., Hoffman, B. D., Brenner, M. D., Zhou, R., Parsons, M., Yang, M. T., et al. (July 2010). Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature, 466*(7303), 263−266.

Guevorkian, K., Gonzalez-Rodriguez, D., Carlier, C., Dufour, S., & Brochard-Wyart, F. (August 2011). Mechanosensitive shivering of model tissues under controlled aspiration. *Proceedings of the National Academy of Sciences of the United States of America, 108*(33), 13387−13392.

Hadorn, M., Boenzli, E., Sørensen, K. T., Fellermann, H., Eggenberger Hotz, P., & Hanczyc, M. M. (December 2012). Specific and reversible DNA-directed self-assembly of oil-in-water emulsion droplets. *Proceedings of the National Academy of Sciences of the United States of America, 109*(50), 20320−20325.

Harris, A. R., Peter, L., Bellis, J., Baum, B., Kabla, A. J., & Charras, G. T. (October 2012). Characterizing the mechanics of cultured cell monolayers. *Proceedings of the National Academy of Sciences of the United States of America, 109*(41), 16449−16454.

Hutson, M. S., Tokutake, Y., Chang, M.-S., Bloor, J. W., Venakides, S., Kiehart, D. P., et al. (April 2003). Forces for morphogenesis investigated with laser microsurgery and quantitative modeling. *Science (New York, N.Y.), 300*(5616), 145−149.

Jorjadze, I., Pontani, L.-L., Newhall, K. A., & Brujic, J. (March 2011). Attractive emulsion droplets probe the phase diagram of jammed granular matter. *Proceedings of the National Academy of Sciences of the United States of America, 108*(11), 4286−4291.

Keese, C., & Giaever, I. (March 1983). Cell growth on liquid microcarriers. *Science, 219*(4591), 1448−1449.

Köster, S., Angilè, F. E., Duan, H., Agresti, J. J., Wintner, A., Schmitz, C., et al. (July 2008). Drop-based microfluidic devices for encapsulation of single cells. *Lab on a Chip, 8*(7), 1110−1115.

Krafft, M. P., & Riess, J. G. (May 2009). Chemistry, physical chemistry, and uses of molecular fluorocarbon−hydrocarbon diblocks, triblocks, and related compounds−unique "apolar" components for self-assembled colloid and interface engineering. *Chemical Reviews, 109*(5), 1714−1792.

Leduc, C., Campàs, O., Zeldovich, K. B., Roux, A., Jolimaitre, P., Bourel-Bonnet, L., et al. (December 2004). Cooperative extraction of membrane nanotubes by molecular motors. *Proceedings of the National Academy of Sciences of the United States of America, 101*(49), 17096−17101.

Legant, W. R., Miller, J. S., Blakely, B. L., Cohen, D. M., Genin, G. M., & Chen, C. S. (November 2010). Measurement of mechanical tractions exerted by cells in three-dimensional matrices. *Nature Methods, 7*(12), 969−971.

Maitre, J. L., Berthoumieux, H., Krens, S. F. G., Salbreux, G., Julicher, F., Paluch, E., et al. (October 2012). Adhesion functions in cell sorting by mechanically coupling the cortices of adhering cells. *Science (New York, NY), 338*(6104), 253−256.

Marchbank, A. (March 1995). Fluorocarbon emulsions. *Perfusion, 10*(2), 67−88.

Mason, T., & Bibette, J. (October 1996). Emulsification in viscoelastic media. *Physical Review Letters, 77*(16), 3481−3484.

Pontani, L.-L., Jorjadze, I., Viasnoff, V., & Brujic, J. (June 2012). Biomimetic emulsions reveal the effect of mechanical forces on cell-cell adhesion. *Proceedings of the National Academy of Sciences of the United States of America, 109*(25), 9839−9844.

Rauzi, M., & Lenne, P.-F. (January 2011). Cortical forces in cell shape changes and tissue morphogenesis. *Current Topics in Developmental Biology, 95*, 93−144.

Riess, J. G. (September 2001). Oxygen carriers ("blood substitutes") − Raison d'Etre, chemistry, and some physiology. *Chemical Reviews, 101*(9), 2797−2920.

Riess, J. G. (October 2009). Highly fluorinated amphiphilic molecules and self-assemblies with biomedical potential. *Current Opinion in Colloid & Interface Science, 14*(5), 294−304.

Riess, J. G., & Krafft, M. P. (August 1998). Fluorinated materials for in vivo oxygen transport (blood substitutes), diagnosis and drug delivery. *Biomaterials, 19*(16), 1529−1539.

Seemann, R., Brinkmann, M., Pfohl, T., & Herminghaus, S. (January 2012). Droplet based microfluidics. Reports on progress in physics. *Physical Society (Great Britain), 75*(1), 016601.

Smith, M. L., Gourdon, D., Little, W. C., Kubow, K. E., Eguiluz, R. A., Luna-Morris, S., et al. (October 2007). Force-induced unfolding of fibronectin in the extracellular matrix of living cells. *PLoS Biology, 5*(10), e268.

Tan, J. L., Tien, J., Pirone, D. M., Gray, D. S., Bhadriraju, K., & Chen, C. S. (February 2003). Cells lying on a bed of microneedles: an approach to isolate mechanical force. *Proceedings of the National Academy of Sciences of the United States of America, 100*(4), 1484−1489.

Teh, S.-Y., Lin, R., Hung, L.-H., & Lee, A. P. (February 2008). Droplet microfluidics. *Lab on a Chip, 8*(2), 198−220.

Trepat, X., Wasserman, M. R., Angelini, T. E., Millet, E., Weitz, D. A., Butler, J. P., et al. (May 2009). Physical forces during collective cell migration. *Nature Physics, 5*(6), 426−430.

Trichet, L., Campàs, O., Sykes, C., & Plastino, J. (March 2007). VASP governs actin dynamics by modulating filament anchoring. *Biophysical Journal, 92*(3), 1081−1089.