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Functionalized Lipid Droplets and Microfluidics approach to study immune cell polarity *in vitro*

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Running title: Immune synapse and cell polarity in a chip

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Abstract

The study of lymphocyte polarization upon antigen encounter typically relies on the random pairing between the cells of interest and a stimulating particle (micro bead) that mimics only some of the properties of the antigen-presenting cells. Here, we show how to build and use a microfluidic chip that allows to multiplex and synchronize the encounter between a lymphocyte and an antigen-presenting object: a functionalized oil-in-water droplet. We also explain how to fabricate and functionalize lipid droplets, an antigen presenting tool that is, at the same time, deformable, fluid and spherical.

Key words Microfluidics, immune synapse, dynamics, live imaging, polarization, lymphocyte

1. Introduction

Immune synapse formation is accompanied both in B and T lymphocytes by a quick cytoplasmic rearrangement that leads to the establishment of a polarity axis passing through the center of the two cells. In order to observe the polarization events that occur with antigen extraction (in B cells), CD4 signaling (in CD4⁺ T cells) and granules release (in cytotoxic T cells), one needs an activating object carrying the

ligands of the specific receptor (BCR or TCR) and a method to control the precise moment when cells are activated and follow the cell in 3D+time with a seconds-to-minute time resolution.

In vivo, lymphocytes are activated by an antigen bearing cell, e.g. a subcapsular sinus macrophage for B cell [1], antigen presenting cells for CD4⁺ T cells [2] or target cells for CD8⁺ T cells [3]. For *in vitro* experiments, to homogenize the results, it is customary to use an object that mimics these cells' geometry, physical and chemical properties, such as engineered cells [4–6] or functionalized micro-beads [7, 8]. A recently introduced antigen presenting tool, functionalized lipid droplets, has sizes comparable to the cellular one, is deformable, and presents the antigen to the cell on a fluid surface (as e.g. lipid bilayers) [9–11]. We describe here how to produce and functionalize these lipid droplets. The development of this protocol is thoroughly described in Refs. [12, 13].

The encounter can be conveniently synchronized and multiplexed in a dedicated microfluidic chip that we designed for this purpose [14], inspired by previous setups [15, 16]. In this method paper, we present how to build and use this microfluidic chip to follow cell activation by microscopy. The chips are produced in PDMS by soft lithography after the design and construction of the mold (which can be reused after the first production step). The fabrication and optimization of the chip have been thoroughly described in Refs. [14, 17].

Ultimately, the experiment consists in loading the droplet in the chip, loading the cells and observe several doublets by live microscopy, possibly using at least two colors to detect simultaneously the kinetic of antigen accumulation (synapse formation) at the kinetic of polarization (e.g. cytoskeleton).

This protocol describes how to (1) microfabricate the master mold (reusable), (2) build the microfluidic chip, (3) formulate and functionalize the lipid droplets, and (4) assemble the system and perform the experiments using as example B lymphocyte cell lines from a B cell lymphoma (IIA1.6). Adaptation to other cell types is possible and will be indicated, when possible, in the protocol.

2. Materials

2.1 Microfabrication

1. Single-side polished silicon wafer, 4-inches, pre-heated for > 30 min (*see Note 1*)
2. Negative tone photoresist, SU-8 2015, Microchem [18]
3. Spin-coater
4. Mask aligner, MJB4 - SÜSS MicroTec - or UV-Kub -Kloe-, turned on 20 min before microfabrication
5. SU-8 developer, Microchem, or PEGDA, CAS 26570-48-9
6. Isopropanol, $\geq 99.5\%$
7. 3 Hot plates, set at 65°C, 95°C and 200°C > 30 min before microfabrication (*see Note 2*).
8. Chrome or plastic mask containing the micro-traps designs (*see Note 3*)
9. O₂ plasma
10. Silane, perfluorodichlorodimethylsilane
11. Vacuum bell jar
12. Pumice stones

2.2 Microfluidic chip assembly

1. PDMS, RTV 615 (*see Note 4*), mix the needed volume at 1:9 ratio, the mixture should have plenty of bubbles. Prepare at the time of experiment (*see Note 5*).
2. Microfluidic traps master (initial wafer or epoxy replica) (*see Notes 3 and 20*)
3. Vacuum bell jar
4. Oven set at > 60°C
5. Scalpel
6. Razor blade (optional)

7. Cutting mat
8. 0.5 mm diameter microfluidic biopsy puncher, with plunger (*see Note 6*)
9. Tape, 3M Scotch 810 magic tape
10. Ethanol >98 %
11. Dry compressed air
12. Disposable syringe, regular tip
13. Flat tweezers
14. Sonicator bath
15. Fluorodish, World Precision instruments FD35-100
16. Atmospheric plasma cleaner, PDC-32G Harrick
17. 0.2 % w/v PVP K90 solution, MilliQ water with 0.2 % PVPK90, wait for dissolution and store up to one month at 4°C. Filter before using.
18. Needle, Stainless Steel Plastic Hub Dispensing Needles 23 GA Kahnetics KDS2312P (*see Note 6*).

2.3 Droplet formulation and functionalization

1. Oil phase: DSPE-PEG(2000) Biotin, 880129, Avanti Lipids (*see Note 7*), 0.05 mg/mL in soybean oil (*see Note 8*). Resuspend DSPE-PEG(2000)-biotin at 10 mg/mL in chloroform. Add 150 μ L of this solution to 30 g of soybean oil, stir gently, and leave in a vacuum bell jar for over 3h30 at room temperature (*see Note 9*).
2. Aqueous phase: 15%w/v Pluronic F68, CAS 9003-11-6 (from 30% stock solution in DI water) + 1%w/v Sodium alginate, CAS 9005-38-3 (from 4%w/v stock in H₂O). Prepare the continuous by adding 2.5 g Sodium alginate 4%w/v with a spatula in a small beaker, then completing to 5 g with deionized water. Add 5 g of Pluronic F68 solution 30%w/v. Stir gently with the spatula, avoiding bubbles (remove bubbles using a vacuum bell once

the solution is prepared).

3. Couette emulsifier, TSR Instrument - Mérignac France
4. PB+Tween20: PB buffer, pH=7, 20mM ionic force (Phosphate buffer) + Tween 20 0.2% v/v stock solution (*see Note 10*)
5. Fluorescent streptavidin stock solution (*see Note 11*)
6. Biotinylated ligand: Biotin-SP AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG, F(ab')₂ fragment specific, Jackson ImmunoResearch 115-066-072, resuspended in H₂O, aliquoted and stored at -80°C (*see Note 12*).
7. 0.60 mL Low-binding microtubes, Axygen MCT-060-L-C

2.4 Microfluidic pairing to reconstitute immune synapses

1. Metal injectors, Stainless Steel Plastic Hub Dispensing Needles 23 GA Kahnetics KDS2312P, kept in ethanol >98% a long time in advance to recover only the metal injector after the glue is degraded (*see Notes 6 and 13*).
2. Medical tubing, Tygon ND 100-80, Saint-Gobain, all pre-cut at the same length for injection.
3. Cell culture media + 25 mM HEPES (*see Note 14*).
4. Fluorescence microscope for live imaging (videomicroscope or spinning disk), in a 37°C cage incubator.
5. 2 (or 3) Pressure controllers, Fluigent LineUp Flow-EZ, with P-CAP-2 mL air-tight caps for reservoir pressurization. Reservoirs for the controllers should be disposable or washable (here, we use Eppendorfs tubes of 1.5 mL or 2 mL), and installed within the 37°C box of the microscope.
6. Lymphocyte cell suspension (*see Note 15*) in cell culture media + 25 mM HEPES, 200

μL /well in a 96-well plate (*see Note 16*).

7. Functionalized droplet suspension prepared in 3.3 (*see Note 17*).

3. Methods

3.1 Microfabrication

This procedure should be performed in a clean room for microfabrication. Here, we describe the microfabrication protocol for designs adapted to trapping single cells of B lymphocyte cell lines (Figure 1), where the microfluidic chip should have a height of $15\ \mu\text{m}$ (*see Note 18*), and using SU-8 2015, 1250 cSt (*see Note 19*). This procedure should be performed once at the beginning of the project, and then the mold can be reused until it degrades.

1. Bake at 200°C for 5 minutes on a hotplate to dehydrate the surface of the 4 inches silicon wafer.
2. Place the silicon wafer on a spin-coater. Pour SU-8 photoresist in the center (approximately 5 mL). Spin the resist for 3 min at 3250 rpm, with an acceleration of 300 rpm/second.
3. Proceed to the soft-bake of the photoresist by heating the SU-8 coated wafer for 3 min on a 95°C hotplate.
4. Place the mask in hard contact with the silicon wafer in the mask aligner. Adjust the exposure time to reach an exposure dose of $140\ \text{mJ}/\text{cm}^2$ (*see Note 20*).
5. Remove the mask, and post bake the coated silicon wafer at 95°C for 4 min.

6. Develop the designs by gently immersing the wafer into SU-8 developer (provided by the manufacturer, or pure PEGDA) for 3 min sharp while slowly rotating. Non-exposed regions should be entirely dissolved.
7. Remove the mold from the PEGDA solution and gently rinse the mold (front and back) with PEGDA for 10 s and then isopropanol for another 10 s (*see Note 21*).
8. Finally, dry the mold (front and back) with dry air pressure.
9. Check the design using binoculars. If it presents cracking, bake it at 200°C for a minute and check it again. Repeat this step until observing homogeneous structures (*see Note 22*).
10. To improve long-term use and storage, coat the wafer with silane: clean the wafer and activate it with an O₂ plasma for 5 min (30 W). Put the wafer in a Petri dish, with 20 µL of silane aside in a small tube top, and pieces of pumice stones in another tube top, under a closed, dedicated vacuum bell jar (saturated atmosphere) for 30 min (*see Note 23*).

[INSERT FIGURE 1 HERE]

3.2 Microfluidic chip assembly

Microfluidic chip preparation should be performed while avoiding dust and keeping everything as clean as possible (rinsing tools with ethanol, changing tools regularly), to avoid damaging the designs. Unless stated otherwise, microfluidic chips are always manipulated with the design face up to avoid crushing, and put on the cutting board.

1. Pour the mixed PDMS onto the microfluidic chip mold. Leave in the vacuum bell until bubbles have disappeared. Cure at 70°C for at least 2 hours. Once baked, the PDMS-filled mold can be kept to do an experiment later.
2. Cut out the microfluidic chips using the scalpel, and cut the sides of the chips to adjust to the size of the bottom of a Fluorodish and to have clean sides, possibly with a razor

blade. PDMS chips can be stored like this, covered with tape and protected from dust, for a few weeks.

3. Make 0.5 mm-diameter holes using the puncher at the inlet/outlet sites of the microfluidic design (*see Note 24*). Rinse the puncher with ethanol between two punches to avoid carrying dust or debris.
4. Flush ethanol into the holes using a syringe and a needle from the design side, then flush from the opposite side while making the needle go through to make sure the hole is well done.
5. With the help of flat tweezers, put the microfluidic chip in a beaker filled with >98 % ethanol, design side down, and sonicate for 5 minutes.
6. Recover the microfluidic chip using the tweezers, dry it using compressed dry air, and clean it with tape. If possible, check the integrity of the designs and the placement of the injection holes using a benchtop magnifier (*see Note 25*).
7. Place the microfluidic chip and the fluorodish, without its cover, in the plasma cleaner for 1 min (follow manufacturer's instructions for manipulating the plasma cleaner, we use it at maximum power i.e. 18W), and then take the chip and add it on the glass bottom of the fluorodish, design facing down. Ensure both sides are in contact by looking at the homogeneity of the color of the contact zone. Put >1 h at 60°C to reinforce bonding.
8. Activate the bonded chip in the plasma cleaner for 1 min before injecting gently with the 0.2 %w/v PVP-K90 solution in MilliQ water using a syringe with a filter, until some solution exited and visual control confirms the whole design is filled (*see Note 26*). Then, add more solution on top to prevent drying and store the dish at 4°C (*see Note 27*).

3.3 Droplet formulation

This is one method of droplet formulation and functionalization that allows to obtain droplets of a diameter comparable to that of a cell, and mobility of the ligand at the surface of the droplet to follow

antigen clustering during immune synapse formation, but other techniques allow to obtain very different droplet characteristics (see **Note 28**).

1. Slowly add the oil phase to the aqueous phase: add 2-3 drops, stir gently with a spatula until the oil is incorporated before adding more drops, and repeat this process. Progressively, the oil phase gets incorporated more easily and can be added faster. During this whole step, avoid introducing air bubbles. A rough, white emulsion should be obtained.
2. Add the emulsion to the Couette cell, and shear at 150 rpm ($e = 100 \mu\text{m}$) (see **Note 29**). Recover the emulsion as it gets out from the Couette cell, and collect it in a tared beaker.
3. The obtained emulsion is at 25% aqueous phase, with 15%w/v Pluronic F68 in it.
4. Adjust the concentrations to obtain a final emulsion at 5% in oil phase, and 1% in Pluronic-F68. Place the emulsion in a separating funnel, protected from light, for 24 h at 1%w/v Pluronic F68, 5%w/v oil phase, then remove the clearer part under the cream of droplet. Wash the emulsion in 1%w/v Pluronic F68 like this at least 2 times, or until the lower part of the solution is clear (see **Note 30**).
5. After the last wash, remove the clear part of the solution, below the decanted emulsion, and discard it. Recover the final emulsion and store it in glass vials at 12°C, protected from light (see **Notes 31 and 32**). This emulsion is used as stock emulsion, and can be used for up to a year, or until the appearance of the emulsion has changed i.e. oil and water are two separated phases.
6. Before starting to do experiments with droplets, take some emulsion, dilute it 100 times and verify their size distribution. Using this protocol, the droplet diameter should be around 9 μm on average, with some dispersion in size.

[INSERT FIGURE 2 HERE]

3.3 Droplet functionalization

Droplet functionalization should be performed on the day of the experiment (Figure 2). Throughout the protocol of droplet functionalization, use only low-binding microtubes. See **Note 28** for an alternative functionalization.

1. Take 2 μL of stock droplet emulsion using a cut pipet tip to not damage the emulsion, and put it into a microtube, before adding 198 μL of PB+Tween 20 buffer.
2. Wash the emulsion: centrifuge the emulsion at 2000 rpm for 30 sec in a minifuge. Remove 180 μL of the aqueous phase (*see Note 33*).
3. Add 180 μL PB+Tween 20. Repeat the washing step (**step 2**) three times (*see Note 34*).
4. After the last wash, leave 20 μL of emulsion in the eppendorf. In another low-binding microtube, prepare 180 μL of PB+Tween 20 buffer with 2.5 μg of streptavidin. Add this solution to the 20 μL of droplet emulsion and homogeneize well.
5. Incubate for 30 min on a rotating wheel at RT, protected from light.
6. Wash droplets as detailed in **steps 2-3** to remove the excess of streptavidin.
7. After the last wash, leave 20 μL of emulsion in the eppendorf. In another low-binding microtube, prepare 180 μL of PB+Tween 20 buffer with 5 μg of biotinylated ligand.
8. Incubate for 30 min on a rotating wheel at RT, protected from light.
9. Wash droplets as detailed in **steps 2-3** to remove the excess of ligand, and leave in 200 μL total (*see Note 35*).

3.4 Immune synapse reconstitution in the microfluidic chip

[INSERT FIGURE 3 HERE]

1. On the morning of the experiment, take the microfluidic chips out of the fridge and to

room temperature.

2. Remove the PVP solution from the fluorodish, and inject the microfluidic chip with cell culture media (with a drug if the experiment involves a treatment, *see Note 36*) until a large drop has formed on the exit side. Add a little bit of media on top of the microfluidic chip to prevent drying. Put the microfluidic chip in an incubator (37°C, 5% CO₂) until the time of experiment (*see Note 37*).
3. Cut 3 pieces of tubing or similar length, long enough to join the reservoir of the pressure controller and the stage of the microscope. Insert a metal injector at the extremity of each piece of tubing, and install two of these tubes on the two pressure controllers.
4. Take a microfluidic chip, remove some of the media on top, and install it on the microscope stage (*see Note 38*). Orient it so that the entry injection point is on the side of the pressure controller and reservoirs.
5. Prepare one eppendorf with the well-resuspended target suspension (*see Note 39*), and one eppendorf with cell culture media + 25 mM HEPES (+ drug). Install them on the reservoir holders.
6. Gradually increase the pressure for the target suspension, until a drop is getting out of the metal injector. Lower the pressure to decrease the flow and prevent losing the content of the reservoir, and deposit the drop on the entry point of the microfluidic chip before inserting the injector in the entry point hole. Only then, increase the pressure to inject the targets (*see Note 40*). Lower the pressure when enough targets have been trapped to avoid more targets getting into the microfluidic chip (Figure 3) (*see Note 41*).
7. Gradually increase the pressure for the cell culture media, until a drop is getting out of the metal injector. Lower the pressure to decrease the flow and prevent losing the content of the reservoir, and deposit the drop on the entry point of the microfluidic chip. Hold the fluorodish and the cell culture media tubing with one hand, remove the target suspension tubing and injector with the other one, and replace it by the cell culture media tubing and

injector (*see Note 42*). Increase the injection pressure to wash the microfluidic chip from all non-trapped targets (Figure 3). Cut the pressure for the target suspension, attach the metal injector at a high point to prevent the remaining liquid in the tubing from flowing out (*see Note 43*). Then, disconnect its tubing and reservoir.

8. While the microfluidic chip is being washed with cell culture media, set up the microscopy settings (*see Note 44*). Look at the fluorescence of the target and adjust the settings, identify the fields of view to image, and add some of the lymphocyte suspension on the side of the fluorodish, next to the microfluidic chip, to adjust settings for fluorescent imaging if they are stained.
9. Install the third tubing + metal injector on the free pressure controller. Prepare an eppendorf with the lymphocyte suspension, and set it up on the pressure controller. As in **step 7**, replace the cell culture media inlet with the lymphocyte suspension inlet. When the injector is in place, keep the pressure low while selecting the fields of view for acquisition.
10. Launch the acquisition, let one image be acquired before increasing the injection pressure of the lymphocyte suspension to have the initial state of the target, and the first time of contact between the target and the lymphocyte (Figure 4). When there are enough target-lymphocyte doublets (*see Note 41*), decrease the injection pressure to prevent more lymphocytes from accumulating.

[INSERT FIGURE 4 HERE]

3.5 Final remarks

The 4D (+ color if necessary) data collected with a confocal or epifluorescence microscope can be reconstructed in a single movie to obtain a multi-tiff file (see Figure 4

for examples of images obtained). To analyze polarization phenomena we recommend to crop, on each field of view, the traps where both a target and a lymphocyte are present, and to remove the time points that precede the arrival of the cell. Image analysis tools (Imaris, FIJI, etc) can then be applied to track, for example, organelles and cytoskeleton dynamics and/or the morphodynamics of the nucleus and the cell.

4. Notes

1. This is to remove humidity/water that might prevent photoresist adhesion.
2. Pre-heat the hot plates to have a stable temperature, and make sure to keep the humidity in the room as low as possible.
3. Various designs have been described over the years [9, 10, 14, 15, 19–21], and can be used for that protocol. Here, we describe the protocol for the design used in [9] and described in [14], with a B lymphocyte cell line. One can also use smaller micro-traps for primary lymphocytes, as shown in Figures 1 and 4 [9]. Photomasks can be ordered via several online services (e.g. <https://www.selba.ch>, <https://www.jd-photodata.co.uk/>, <https://www.microlitho.co.uk/>)
4. We find that the other commonly-used PDMS, Sylgard, has other mechanical properties and tends to break more easily than RTV during the repeated injections with needles. Of note, RTV and Sylgard may differ in adsorbing drugs during experiments.
5. The PDMS components can be mixed in a plastic cup, or a falcon tube, mixed using a disposable pipette. The PDMS mix can also be prepared in advance, in excess, and stored at -20°C in a falcon tube. In that case, bubbles will disappear over time, and the tube should be put back to room temperature a little before using, but the PDMS-filled molds will not need to be left very long in the vacuum bell.
6. One can also use a 0.75 mm diameter microfluidic puncher, and replace the metal

injectors and needles by PTFE tubing by making a Tygon-PTFE junction.

7. This can also be replaced by DOPE, or other functionalization of DSPE-PEG.
8. One can also use other oils, such as mineral oil, to achieve different droplet properties.
9. It is crucial that the chloroform has well evaporated, otherwise this changes the viscosity of the solution and will affect the final droplet size.
10. Keep a stock of PB buffer, pH=7, 20 mM, and a stock of PB+Tween 20 20% v/v, to prepare the 0.2 % solution on the day of the experiment.
11. We tried different providers, fluorophores, and stock concentrations for the streptavidin, and all were compatible with our system.
12. This ligand can be changed depending on the experiment. We have also used biotinylated BSA as a negative control, F(ab')₂ anti mouse IgM or biotinylated HEL for primary B cells, biotinylated ICAM-1, biotin anti-CD3 for primary T lymphocytes. These ligands can also be mixed to have several functions on the droplets, although their proportions would have to be calibrated.
13. Metal injectors can also be custom-made, but we find that this is the most cost-effective protocol. Metal injectors can be re-used between experimental days, but need to be flushed with MilliQ water after an experiment, kept in ethanol, and sonicated before being reused. This avoids the presence of dried cells or medium components, and therefore debris or clogging.
14. During the experiment, the chip is constantly connected to the pressure controller via the metal injector and tubing, preventing us from adding the CO₂ control cover on top. We find that adding 25 mM HEPES and keeping the whole microscope at 37°C keeps the cells healthy for the duration of the experiment (1 h maximum in our case).
15. We find that the concentration of the lymphocyte cell suspension depends on the cell type and version of the microfluidic design used. Typically, for the lymphocyte cell line, we use $1.5 \cdot 10^6$ cells/mL, and for primary murine lymphocytes, we use $3 \cdot 10^6$ cells/mL.

16. We use one well/chip. We find that it is better to separate the cells in wells so as to prevent repetitive resuspension and manipulation of the same cells, and facilitate addition of drugs for pre-incubation in a systematic manner during the day of the experiment. Overall, this improves reproducibility.
17. Here, we use antigen-coated oil droplets to activate B lymphocytes. However, any object of similar dimension such as beads or other cells can be used. The concentration of this solution should be tuned to the size of the object. In experiments using beads or droplets, and involving drug treatment, we advise to resuspend the beads/droplets with cell culture media + 25 mM HEPES + drugs. Of note, under a certain concentration of droplets, we found that almost nothing was trapped.

In our case, we use antigen-coated droplets on which the antigen is mobile, in order to visualize antigen recruitment and follow immune synapse formation.
18. This protocol should be adapted for smaller cells, e.g. primary cells, where not only the trap design is modified, but also the height of the microfluidic chip should be close to 8 μm to avoid cells going above or below droplets, or being stacked on top of each other.
19. Viscosity of the SU-8 varies between lots and with aging of the resin, so this protocol, and in particular spin-coating (time, speed) of the SU-8 photoresist in step 2, should be adapted to the SU-8 batch to control final thickness of mold. It is good practice to aliquot the stock resin solution in 50-100 mL bottles to limit aging.
20. For a source having a power of 20 mW/cm^2 , the exposure time is calculated by dividing the dose by the lamp power, which gives an exposure time of 7.0 s.
21. If the wafer takes a whitish aspect when put in contact with isopropanol, that means that the development is not complete, and you should increase the time or refresh the developer solution.
22. If at this stage, the patterns are not well resolved, or you see detachment, oblique sidewalls, etc., you have to consider optimizing the exposure and development steps.

23. This step improves long-term use and storage of wafers, and facilitates the detachment of cured PDMS from the wafer. We advise frequent users to prepare epoxy replicates of the microfluidic chips when possible (depending on size and design resolution), thus as to avoid using the initial wafers on a day-to-day basis as they are very fragile. An example of well adapted protocol for it can be found in Refs. [22, 23]. Of note for chips with small patterns the procedure might not work.
24. Visualizing the injection sites can be made easier by putting ethanol on the chip, which will be retained on the designs.
25. The cleaning procedures of the microfluidic chip can vary depending on the environment they are prepared in. Here, we propose a protocol for chips prepared in a cleanroom-free facility, and some steps could be eliminated or simplified in a microfluidics-dedicated space, with less dust.
26. PDMS is initially very hydrophobic, making it difficult to inject liquid in it and avoid bubbles. Filling the chip with the PVP solution allows a coating of the PDMS, and its permanent contact with an aqueous solution, keeping it more hydrophilic until the time of the experiment.
27. Microfluidic chips can be kept at 4°C in the 0.2 %w/v PVP K90-filled fluorodish for up to a week before the experiment.
28. Of note we tested two types of functionalization: one where the biotinylated phospholipids are mixed with the oil (bulk) and one where they are incorporated in the droplet after droplet fabrication (surface) [10]. We present here only the bulk one, as it is the one that permits the accumulation of the antigen at the synapse. Surface-functionalized droplets do not show antigen accumulation, but allow, nevertheless, the activation of the cells. This functionalization technique allows very precise quantification of the ligands present at the interface and is less demanding in terms of materials. The reader could be also interested in changing the mechanical properties of the droplets,

which is doable modifying the oil mixture. Details on alternative functionalization methods and surface tension can be found in [10] and [24].

29. The rough emulsion is sheared in the Couette cell to obtain more homogeneous and smaller droplets. Changing the speed/settings of the Couette cell, or the viscosity of the water phase will change the average size of the droplets.
30. These steps allow to remove some of the smallest droplets, which will not get decanted to the surface of the funnel, and obtain a more homogeneous emulsion.
31. Separate the stock into several glass vials, as repeated openings of the vial will degrade the emulsion faster. This way, the emulsion stock can be used for longer.
32. To store products at 12°C, we find that the most cost-effective item is a small wine cellar for service, where the door can be covered with aluminum foil to block the light.
33. We encourage you to leave the tube in the centrifuge for at least 30 s after the spin so that the emulsion is well concentrated at the surface, on one side. To remove the aqueous phase, use a gel-loading pipet tip and insert it on the side of the eppendorf with the least emulsion, then aspirate slowly the aqueous phase sitting below.
34. During these washes, the Pluronic F68 surfactant in continuous phase is removed. At the oil/water interface, Tween 20 surfactant is progressively added to ensure a homogeneous protein coating.
35. For experiments using drugs, we separate this solution into several eppendorfs, and resuspend the droplets in media+drug to ensure the good equilibrium of drug concentration.
36. PDMS is very permeable, and can adsorb drugs. It is therefore important to pre-incubate the microfluidic chip with cell culture media + drug to obtain a stable concentration of drug treatment during the experiment.
37. Equilibrating the microfluidic chip in an incubator allows it to be at the right temperature and gaz (CO₂) content for the experiment, and therefore maintain good conditions for the

cell as well as for imaging, as temperature differences increase the risk of drifting during time-lapse microscopy.

38. Do not dry the surface of the chip, and do not let it with little media for a long time. The surface should not dry, because this increases the risk of introducing air bubbles into the microfluidic chip when injecting, and of dry media debris entering and damaging the microfluidic chip.
39. We find that it is better to not put all target suspension in the eppendorf at once, but rather add some more for each microfluidic experiment during the day. Indeed, in case of a problem with the pressure controller, one could lose the full content of the eppendorf. In addition, this allows the use of a suspension from the same fresh and clean stock for each acquisition.
40. With our design, our pressure controller, and when putting the reservoir on the side of the microscope stage at a height close to the one of the microfluidic chip, we find that setting the pressure around 12 mbar until liquid gets out of the injector, lowering the pressure to 8-9 mbar while inserting the metal injector into the hole, then injecting targets/lymphocytes with a pressure around 20 mbars, and setting to a resting pressure (very low flow to prevent arrival of more targets/lymphocytes) around 6-8 mbars (this can vary a lot depending on the design and the length of the tube) is a good starting point.
41. A good rule of thumb is that if the microscope settings allow to image 30 traps, then having 10-15 traps with a target/lymphocyte in it is enough (keeping in mind the field of view of the microscope). One should keep in mind that more targets/lymphocytes will certainly arrive and be trapped in the next few minutes, during the wash with cell culture media, and that if there are traps containing several targets/lymphocytes, analysis will be compromised.
42. This manipulation is critical and will be repeated in the next step. It requires some training before being able to avoid injecting air bubbles, which can be mostly prevented

by ensuring that the incoming metal injector has media going out of it. Another risk is to trigger backflow and have targets flowing back to the entry.

43. We find that taping it to the ceiling of the 37°C box of the microscope is the most convenient.
44. As an example of imaging settings, with a spinning disk confocal microscope, we could do 40 minutes acquisitions with 1 image every 30 s, at 3 positions, in 3 colors (Transmission + 2 fluorescent channels), and 21 z slices, using a spinning disk confocal microscope and streaming each channel. For the cell line microfluidic chip design, the Nikon perfect focus system does not work, but one can use the software autofocus on the transmission channel at each time point, and an offset on the z-position for the fluorescent channels.

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Figure captions

Figure 1: (a) Bright field image of one of the chambers of the microfluidic chip designed for lymphocyte cell lines. Each microfluidic chip has four chambers in parallel, each containing a large array of microfluidic traps. Scale bar 100 μm . (b) (Left) Microfluidic traps designed for the B lymphocyte cell line (diameter $\sim 14 \mu\text{m}$) and the (Right) design adapted to primary lymphocytes (diameter $\sim 7 \mu\text{m}$). Scale bar 10 μm .

Figure 2: Functionalization of oil droplets. The stock emulsion is prepared as described in section 3.3 Droplet formulation. Oil droplets presenting biotinylated phospholipids at their surface are sequentially functionalized with a fluorescent streptavidin and a biotinylated ligand, here a biotinylated BCR ligand acting as antigen. Images of diluted, functionalized droplet suspension by spinning disk confocal microscopy below. Scale bar 20 μm .

Figure 3: Steps for immune synapse reconstitution in the microfluidic chip.

Figure 4: Examples of images obtained using this system, on a spinning disk confocal microscope. (a) B lymphocyte cell line (IIA1.6) stained with Hoechst to visualize the nucleus, in contact with an antigen-coated droplet. (b) Naive primary murine B lymphocyte expressing LifeAct-GFP, stained with Hoechst to visualize the nucleus, in contact with an antigen-coated droplet. Illustrations are z-projections over a few planes at the center of the cell, from z-stacks covering the full depth of the cell. Scale bar 5 μm .

Functionalized oil droplets and microfluidics approach to study immune cell polarity in vitro

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Running title: Immune synapse and cell polarity in a chip

Abstract

The study of lymphocyte polarization upon antigen encounter typically relies on the random pairing between the cells of interest and a stimulating particle (micro bead) that mimics only some of the properties of the antigen-presenting cells. Here, we show how to build and use a microfluidic chip that allows to multiplex and synchronize the encounter between a lymphocyte and an antigen-presenting object: a functionalized oil-in-water droplet. We also explain how to fabricate and functionalize lipid droplets, an antigen presenting tool that is, at the same time, deformable, fluid and spherical.

Key words Microfluidics, immune synapse, dynamics, live imaging, polarization, lymphocyte

1. Introduction

Immune synapse formation is accompanied both in B and T lymphocytes by a quick cytoplasmic rearrangement that leads to the establishment of a polarity axis passing through the center of the two cells. In order to observe the polarization events that occur with antigen extraction (in B cells), CD4 signaling (in CD4⁺ T cells) and granules release (in cytotoxic T cells), one needs an activating object carrying the ligands of the specific receptor (BCR or TCR) and a method to control the precise moment when cells are activated and follow the cell in 3D+time with a seconds-to-minute time resolution.

In vivo, lymphocytes are activated by an antigen bearing cell, e.g. a subcapsular sinus macrophage for B cell [1], antigen presenting cells for CD4⁺ T cells [2] or target cells for CD8⁺ T cells [3]. For *in vitro* experiments, to homogenize the results, it is customary to use an object that mimics these cells' geometry, physical and chemical properties, such as engineered cells [4–6] or functionalized micro-beads [7, 8]. A recently introduced antigen presenting tool, functionalized lipid droplets, has sizes comparable to the cellular one, is deformable, and presents the antigen to the cell on a fluid surface (as e.g. lipid bilayers) [9–11]. We describe here how to produce and functionalize these lipid droplets.

The encounter can be conveniently synchronized and multiplexed in a dedicated microfluidic chip that we designed for this purpose [12], inspired by previous setups [13, 14]. In this method paper, we present how to build and use this microfluidic chip to follow cell activation by microscopy. The chips are produced in PDMS by soft lithography after the design and construction of the mold (which can be reused after the first production step).

Ultimately, the experiment consists in loading the droplet in the chip, loading the cells and observe several doublets by live microscopy, possibly using at least two colors to detect simultaneously the kinetic of antigen accumulation (synapse formation) at the kinetic of polarization (e.g. cytoskeleton).

This protocol describes how to (1) microfabricate the master mold (reusable), (2) build the microfluidic chip, (3) formulate and functionalize the lipid droplets, and (4) assemble the system and perform the experiments using as example B lymphocyte cell lines from a B cell lymphoma (IIA1.6). Adaptation to other cell types is possible and marked along the protocol.

2. Materials

2.1 Microfabrication

1. Silicon wafer (4-inch large), pre-heated for > 30min (*see Note 1*)
2. Photoresist, SU-8 2015

3. Spin-coater
4. Mask aligner, MJB4, turned on 20 min before microfabrication
5. SU-8 developer, PEGDA
6. Isopropanol
7. Hot plates, set at 95°C > 30 min before microfabrication (*see Note 2*).
8. Chrome or plastic mask containing the micro-traps designs (*see Note 3*)
9. Oven set at 200°C
10. O₂ plasma
11. Silane
12. Vacuum bell
13. Pumice stones

2.2 Microfluidic chip assembly

1. PDMS, RTV 615 (*see Note 4*), mix the needed volume at 1:9 ratio, the mixture should have plenty of bubbles. Prepare at the time of experiment (*see Note 5*).
2. Microfluidic traps mold (initial wafer or epoxy replica) (*see Note 3*)
3. Vacuum bell
4. Oven set at > 60°C
5. Scalpel
6. Razor blade (optional)
7. Cutting mat
8. 0.5 mm diameter microfluidic puncher (*see Note 6*)
9. Tape, 3M Scotch 810 magic tape
10. Ethanol >98 %
11. Compressed air
12. Syringe

13. Flat tweezers
14. Sonicator bath
15. Fluorodish, World Precision instruments FD35
16. Plasma cleaner, PDC-32G Harrick
17. 0.2 % w/v PVP K90 solution, MilliQ water with 0.2 % PVPK90, wait for dissolution and store up to one month at 4°C. Filter before using.
18. Needle, Stainless Steel Plastic Hub Dispensing Needles 23 GA Kahnetics KDS2312P (*see Note 6*).

2.3 Droplet formulation and functionalization

1. Oil phase: DSPE-PEG(2000) Biotin, Avanti Lipids (*see Note 7*), 0.05 mg/mL in soybean oil (*see Note 8*). Resuspend DSPE-PEG(2000)-biotin at 10 mg/mL in chloroform. Add 150 μ L of this solution to 30 g of soybean oil, stir gently, and leave in a vacuum bell for over 3h30 (*see Note 9*).
2. Aqueous phase: 15%w/v Pluronic F68 (from 30% stock) + 1%w/v Sodium alginate (from 4%w/v stock in H₂O). Prepare by adding 2.5 g Sodium alginate 4%w/v with a spatula in a small beaker, then completing to 5 g with deionized water. Add 5 g of Pluronic F68 solution 30%w/v. Stir gently with the spatula, avoiding bubbles (remove bubbles using a vacuum bell once the solution is prepared).
3. Couette device
4. PB+Tween20: PB buffer, pH=7, 20mM ionic force (Phosphate buffer) + Tween 20 0.2% v/v stock solution (*see Note 10*)
5. Fluorescent streptavidin stock solution (*see Note 11*)

6. Biotinylated ligand: Biotin-SP AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG, F(ab')₂ fragment specific, Jackson ImmunoResearch 115-066-072, resuspended in H₂O, aliquoted and stored at -80°C (*see Note 12*).
7. 0.60 mL Low-binding microtubes, Axygen MCT-060-L-C

2.4 Microfluidic pairing to reconstitute immune synapses

1. Metal injectors, Stainless Steel Plastic Hub Dispensing Needles 23 GA Kahnetics KDS2312P, kept in ethanol >98% a long time in advance to recover only the metal injector after the glue is degraded (*see Notes 6 and 13*).
2. Medical tubing, Tygon ND 100-80, Saint-Gobain, all pre-cut at the same length for injection.
3. Cell culture media + 25 mM HEPES (*see Note 14*).
4. Fluorescence microscope for live imaging (videomicroscope or spinning disk), in a 37°C box.
5. 2 (or 3) Pressure controllers, Fluigent LineUp Flow-EZ, with P-CAP-2 mL air-tight caps for reservoir pressurization. Reservoirs for the controllers should be disposable or washable (here, we use eppendorfs), and installed within the 37°C box of the microscope.
6. Lymphocyte cell suspension (*see Note 15*) in cell culture media + 25 mM HEPES, 200 µL/well in a 96-well plate (*see Note 16*).
7. Functionalized droplet suspension prepared in 3.3 (*see Note 17*).

3. Methods

3.1 Microfabrication

This procedure should be performed in a clean room for microfabrication. Here, we describe the microfabrication protocol for designs adapted to trapping single cells of B lymphocyte cell lines (Figure 1), where the microfluidic chip should have a height of 15 μm (see **Note 18**), and using SU-8 2015, 1250 cSt (*see Note 19*). This procedure should be performed once at the beginning of the project, and then the mold can be reused until it degrades.

1. Bake a 4-inch silicon wafer for 3 min.
2. Place silicon wafer on a spin-coater. Pour SU-8 photoresist in the center and rotate for 3 min at 3250 rpm for 45 sec.
3. Place the wanted mask in hard contact with the silicon wafer in the MJB4 mask aligner. Expose at 140 mJ/cm^2 .
4. Remove the mask, and post bake the coated silicon wafer at 95°C for 4 min.
5. Develop designs: gently immerse the mold into SU-8 developer (PEGDA) for 3 min sharp while slowly rotating. Non-exposed regions should be entirely dissolved.
6. Remove the mold from the PEGDA solution and gently rinse the mold (front and back) with PEGDA for 10 s and then isopropanol for another 10 s. Finally, dry the mold (front and back) with air pressure.
7. Check the design using binoculars. If it presents cracking, bake it at 200°C for a minute and check it again. Repeat this step until observing homogeneous structures.
8. To improve long-term use and storage, the mold is coated with perfluoro-silane: clean wafer and activate it with an O_2 plasma for 5 min (30 W). Put the wafer with 20 μL of silane and pieces of pumice stones under a closed, dedicated vacuum bell (saturated atmosphere) for 30 min (*see Note 20*).

[INSERT FIGURE 1 HERE]

3.2 Microfluidic chip assembly

Microfluidic chip preparation should be performed while avoiding dust and keeping everything as clean as possible (rinsing tools with ethanol, changing tools regularly), to avoid damaging the designs. Unless stated otherwise, microfluidic chips are always manipulated with the design face up to avoid crushing, and put on the cutting board.

1. Pour the mixed PDMS onto the microfluidic chip mold. Leave in the vacuum bell until bubbles have disappeared. Cook at $> 60^{\circ}\text{C}$. Once cooked, the PDMS-filled mold can be kept to do an experiment later.
2. Cut out the microfluidic chips using the scalpel, and cut the sides of the chips to adjust to the size of the bottom of a fluorodish and to have clean sides, possibly with a razor blade. PDMS chips can be stored like this, covered with tape and protected from dust, for a few weeks.
3. Make 0.5 mm-diameter holes using the puncher at the entry-exit sites of the microfluidic design (*see Note 21*). Rinse the puncher with ethanol between two punches to avoid carrying dust or debris.
4. Flush ethanol into the holes using a syringe and a needle from the design side, then flush from the opposite side while making the needle go through to make sure the hole is well done.
5. With the help of flat tweezers, put the microfluidic chip in a beaker filled with $>98\%$ ethanol, design side down, and sonicate for 5 minutes.

6. Recover the microfluidic chip using the tweezers, dry it using compressed air, and clean it with tape. If possible, check the integrity of the designs and the placement of the injection holes using a benchtop magnifier (*see Note 22*).
7. Place the microfluidic chip and the fluorodish, without its cover, in the plasma cleaner for 1 min (follow manufacturer's instruction for manipulating the plasma cleaner), and then take the chip and add it on the glass bottom of the fluorodish, design facing down. Ensure both sides are in contact by looking at the homogeneity of the color of the contact zone. Put >1 h at 60°C to reinforce bonding.
8. Activate the bonded chip in the plasma cleaner for 1 min before injecting gently with the 0.2 % w/v PVP-K90 solution in MilliQ water using a syringe with a filter, until some solution exited and visual control confirms the whole design is filled (*see Note 23*). Then, add more solution on top to prevent drying and store the dish at 4°C (*see Note 24*).

3.3 Droplet formulation

This is one method of droplet formulation and functionalization that allows to obtain droplets of a diameter comparable to that of a cell, and mobility of the ligand at the surface of the droplet to follow antigen clustering during immune synapse formation, but other techniques allow to obtain very different droplet characteristics (*see Note 25*).

1. Slowly add the oil phase to the aqueous phase: add 2-3 drops, stir gently with a spatula until the oil is incorporated before adding more drops, and repeat this process. Progressively, the oil phase gets incorporated more easily and can be added faster. During this whole step, avoid introducing air bubbles. A rough, white emulsion should be obtained.
2. Add the emulsion to the Couette cell, and shear at 150 rpm ($e = 100 \mu\text{m}$) (*see Note 26*). Recover the emulsion as it gets out from the Couette cell, and collect it in a tared beaker.
3. The obtained emulsion is at 25% aqueous phase, with 15%w/v Pluronic F68 in it.

4. Adjust the concentrations to obtain a final emulsion at 5% in oil phase, and 1% in Pluronic-F68. Place the emulsion in a separating funnel, protected from light, for 24 h at 1%w/v Pluronic F68, 5%w/v oil phase, then remove the clearer part under the cream of droplet. Wash the emulsion in 1%w/v Pluronic F68 like this at least 2 times, or until the lower part of the solution is clear (*see Note 27*).
5. After the last wash, remove the clear part of the solution, below the decanted emulsion, and discard it. Recover the final emulsion and store it in glass vials at 12°C, protected from light (*see Notes 28 and 29*). This emulsion is used as stock emulsion, and can be used for up to a year, or until the appearance of the emulsion has changed.
6. Before starting to do experiments with the droplets, take some emulsion, dilute it 100 times and verify the size distribution of the droplets. Using this protocol, the droplet diameter should be around 9 μm on average, with some dispersion in size.

[INSERT FIGURE 2 HERE]

3.3 Droplet functionalization

Droplet functionalization should be performed on the day of the experiment (Figure 2). Throughout the protocol of droplet functionalization, use only low-binding microtubes. See **Note 25** for an alternative functionalization.

1. Take 2 μL of stock droplet emulsion using a cut pipet tip to not damage the emulsion, and put it into a microtube, before adding 198 μL of PB+Tween 20 buffer.
2. Wash the emulsion: centrifuge the emulsion at 2000 rpm for 30 sec in a minifuge. Remove 180 μL of the aqueous phase (*see Note 30*).
3. Add 180 μL PB+Tween 20. Repeat the washing step (**step 2**) three times (*see Note 31*).

4. After the last wash, leave 20 μL of emulsion in the eppendorf. In another low-binding microtube, prepare 180 μL of PB+Tween 20 buffer with 2.5 μg of streptavidin. Add this solution to the 20 μL of droplet emulsion and homogeneize well.
5. Incubate for 30 min on a rotating wheel at RT, protected from light.
6. Wash droplets as detailed in **steps 2-3** to remove the excess of streptavidin.
7. After the last wash, leave 20 μL of emulsion in the eppendorf. In another low-binding microtube, prepare 180 μL of PB+Tween 20 buffer with 5 μg of biotinylated ligand.
8. Incubate for 30 min on a rotating wheel at RT, protected from light.
9. Wash droplets as detailed in **steps 2-3** to remove the excess of ligand, and leave in 200 μL total (*see Note 32*).

3.4 Immune synapse reconstitution in the microfluidic chip

[INSERT FIGURE 3 HERE]

1. On the morning of the experiment, take the microfluidic chips out of the fridge and to room temperature.
2. Remove the PVP solution from the fluorodish, and inject the microfluidic chip with cell culture media (with a drug if the experiment involves a treatment, *see Note 33*) until a large drop has formed on the exit side. Add a little bit of media on top of the microfluidic chip to prevent drying. Put the microfluidic chip in an incubator (37°C, 5% CO₂) until the time of experiment (*see Note 34*).
3. Cut 3 pieces of tubing or similar length, long enough to join the reservoir of the pressure controller and the stage of the microscope. Insert a metal injector at the extremity of each piece of tubing, and install two of these tubes on the two pressure controllers.

4. Take a microfluidic chip, remove some of the media on top, and install it on the microscope stage (*see Note 35*). Orient it so that the entry injection point is on the side of the pressure controller and reservoirs.
5. Prepare one eppendorf with the well-resuspended target suspension (*see Note 36*), and one eppendorf with cell culture media + 25mM HEPES (+ drug). Install them on the reservoir holders.
6. Gradually increase the pressure for the target suspension, until a drop is getting out of the metal injector. Lower the pressure to decrease the flow and prevent losing the content of the reservoir, and deposit the drop on the entry point of the microfluidic chip before inserting the injector in the entry point hole. Only then, increase the pressure to inject the targets (*see Note 37*). Lower the pressure when enough targets have been trapped to avoid more targets getting into the microfluidic chip (Figure 3) (*see Note 38*).
7. Gradually increase the pressure for the cell culture media, until a drop is getting out of the metal injector. Lower the pressure to decrease the flow and prevent losing the content of the reservoir, and deposit the drop on the entry point of the microfluidic chip. Hold the fluorodish and the cell culture media tubing with one hand, remove the target suspension tubing and injector with the other one, and replace it by the cell culture media tubing and injector (*see Note 39*). Increase the injection pressure to wash the microfluidic chip from all non-trapped targets (Figure 3). Cut the pressure for the target suspension, attach the metal injector at a high point to prevent the remaining liquid in the tubing from flowing out (*see Note 40*). Then, disconnect its tubing and reservoir.
8. While the microfluidic chip is being washed with cell culture media, set up the microscopy settings (*see Note 41*). Look at the fluorescence of the target and adjust the settings, identify the fields of view to image, and add some of the lymphocyte suspension on the side of the fluorodish, next to the microfluidic chip, to adjust settings for fluorescent imaging if they are stained.

9. Install the third tubing + metal injector on the free pressure controller. Prepare an eppendorf with the lymphocyte suspension, and set it up on the pressure controller. As in **step 7**, replace the cell culture media inlet with the lymphocyte suspension inlet. When the injector is in place, keep the pressure low while selecting the fields of view for acquisition.
10. Launch the acquisition, let one image be acquired before increasing the injection pressure of the lymphocyte suspension to have the initial state of the target, and the first time of contact between the target and the lymphocyte (Figure 4). When there are enough target-lymphocyte doublets (*see Note 38*), decrease the injection pressure to prevent more lymphocytes from accumulating.

[INSERT FIGURE 4 HERE]

3.5 Final remarks

The 4D (+ color if necessary) data collected with a confocal or epifluorescence microscope can be reconstructed in a single movie to obtain a multi-tiff file (see Figure 4 for examples of images obtained). To analyze polarization phenomena we recommend to crop, on each field of view, the traps where both a target and a lymphocyte are present, and to remove the time points that precede the arrival of the cell. Image analysis tools (Imaris, FIJI, etc) can then be applied to track, for example, organelles and cytoskeleton dynamics and/or the morphodynamics of the nucleus and the cell.

4. Notes

1. This is to remove humidity/water that might prevent photoresist adhesion.

2. Pre-heat the hot plates to have a stable temperature, and make sure to keep the humidity in the room as low as possible.
3. Various designs have been described over the years [9, 10, 12, 13, 15–17], and can be used for that protocol. Here we describe the protocol for the design used in [9] and described in [12], with a B lymphocyte cell line. One can also use smaller micro-traps for primary lymphocytes, as shown in Figures 1 and 4 [9].
4. We find that the other commonly-used PDMS, Sylgard, has other mechanical properties and tends to break more easily than RTV during the repeated injections with needles.
5. The PDMS mix can also be prepared in advance, in excess, and stored at -20°C in a falcon tube. In that case, bubbles will disappear over time, and the tube should be put back to room temperature a little before using, but the PDMS-filled molds will not need to be left very long in the vacuum bell.
6. One can also use a 0.75 mm diameter microfluidic puncher, and replace the metal injectors and needles by PTFE tubing by making a Tygon-PTFE junction.
7. This can also be replaced by DOPE, or other functionalization of DSPE-PEG.
8. One can also use other oils such as mineral oil, FC-40, lipiodol oil, to achieve different droplet properties.
9. It is crucial that the chloroform has well evaporated, otherwise this changes the viscosity of the solution and will affect the final droplet size.
10. Keep a stock of PB buffer, $\text{pH}=7$, 20 mM, and a stock of PB+Tween 20 20% v/v, to prepare the 0.2 % solution on the day of the experiment.
11. We tried different providers, fluorophores, and stock concentrations for the streptavidin, and all were compatible with our system.
12. This ligand can be changed depending on the experiment. We have also used biotinylated BSA as a negative control, F(ab')_2 anti mouse IgM or biotinylated HEL for primary B cells, biotinylated ICAM-1, biotin anti-CD3 for primary T lymphocytes. These ligands

can also be mixed to have several functions on the droplets, although their proportions would have to be calibrated.

13. Metal injectors can also be custom-made, but we find that this is the most cost-effective protocol. Metal injectors can be re-used between experimental days, but need to be flushed with MilliQ water after an experiment, kept in ethanol, and sonicated before being reused. This avoids the presence of dried cells or medium components, and therefore debris or clogging.
14. During the experiment, the chip is constantly connected to the pressure controller via the metal injector and tubing, preventing us from adding the CO₂ control cover on top. We find that adding 25 mM HEPES and keeping the whole microscope at 37°C keeps the cells healthy for the duration of the experiment.
15. We find that the concentration of the lymphocyte cell suspension depends on the cell type and version of the microfluidic design used. Typically, for the lymphocyte cell line, we use $1.5 \cdot 10^6$ cells/mL, and for primary murine lymphocytes, we use $3 \cdot 10^6$ cells/mL.
16. We use one well/chip. We find that it is better to separate the cells in wells so as to prevent repetitive resuspension and manipulation of the same cells, and facilitate addition of drugs for pre-incubation in a systematic manner during the day of the experiment. Overall, this improves reproducibility.
17. Here, we use antigen-coated oil droplets to activate B lymphocytes. However, any object of similar dimension such as beads or other cells can be used. The concentration of this solution should be tuned to the size of the object. In experiments using beads or droplets, and involving drug treatment, we advise to resuspend the beads/droplets with cell culture media + 25 mM HEPES + drugs. Of note, under a certain concentration of droplets, we found that almost nothing was trapped.

In our case, we use antigen-coated droplets on which the antigen is mobile, in order to visualize antigen recruitment and follow immune synapse formation.

18. This protocol should be adapted for smaller cells, e.g. primary cells, where not only the trap design is modified, but also the height of the microfluidic chip should be close to 8 μm to avoid cells going above or below droplets, or being stacked on top of each other.
19. Viscosity of the SU-8 can vary between lots, so this protocol, and in particular spinning of the SU-8 photoresist in step 2, should be adapted to the SU-8 batch to control thickness.
20. This step improves long-term use and storage of wafers. We advise frequent users to prepare epoxy replica of the microfluidic chips when possible, so as to avoid using the initial wafers on a day-to-day basis as they are very fragile.
21. Visualizing the injection sites can be made easier by putting ethanol on the chip, which will be retained on the designs.
22. The cleaning procedures of the microfluidic chip can vary depending on the environment they are prepared in. Here, we propose a protocol for chips prepared in a cleanroom-free facility, and some steps could be eliminated or simplified in a microfluidics-dedicated space, with less dust.
23. PDMS is initially very hydrophobic, making it difficult to inject liquid in it and avoid bubbles. Filling the chip with the PVP solution allows a coating of the PDMS, and its permanent contact with an aqueous solution, keeping it more hydrophilic until the time of the experiment.
24. Microfluidic chips can be kept at 4°C in the 0.2 % w/v PVP K90-filled fluorodish for up to a week before the experiment.
25. Of note we tested two types of functionalization: one where the biotinylated phospholipids are mixed with the oil (bulk) and one where they are incorporated in the droplet after droplet fabrication (surface) [10]. We present here only the bulk one, as it is the one that makes the interface more fluid and permits the accumulation of the antigen at

the synapse. Surface-functionalized droplets do not show antigen accumulation, but allow, nevertheless, the activation of the cells. This functionalization technique allows very precise quantification of the ligands present at the interface and is less demanding in terms of materials. The reader could be also interested in changing the mechanical properties of the droplets, which is doable modifying the oil mixture. Details on alternative functionalization methods and surface tension can be found in [10] and [18].

26. The rough emulsion is sheared in the Couette cell to obtain more homogeneous, smaller droplets. Changing the speed/settings of the Couette cell, or the viscosity of the oil phase will change the average size of the droplets.
27. These steps allow to remove some of the smallest droplets, which will not get decanted to the surface of the funnel, and obtain a more homogeneous emulsion.
28. Separate the stock into several glass vials, as repeated openings of the vial will degrade the emulsion faster. This way, the emulsion stock can be used for longer.
29. To store products at 12°C, we find that the most cost-effective item is a small wine cellar for service, where the door can be covered with aluminum foil to block the light.
30. We encourage you to leave the tube in the centrifuge for at least 30 s after the spin so that the emulsion is well concentrated at the surface, on one side. To remove the aqueous phase, use a gel-loading pipet tip and insert it on the side of the eppendorf with the least emulsion, then aspirate slowly the aqueous phase sitting below.
31. During these washes, the Pluronic F68 surfactant in continuous phase is removed. At the oil/water interface, Tween 20 surfactant is progressively added to ensure a homogeneous protein coating.
32. For experiments using drugs, we separate this solution into several eppendorfs, and resuspend the droplets in media+drug to ensure the good equilibrium of drug concentration.

33. PDMS is very permeable, and can absorb drugs. It is therefore important to pre-incubate the microfluidic chip with cell culture media + drug to obtain a stable concentration of drug treatment during the experiment.
34. Equilibrating the microfluidic chip in an incubator allows it to be at the right temperature and gaz (CO₂) content for the experiment, and therefore maintain good conditions for the cell as well as for imaging, as temperature differences increase the risk of drifting during time-lapse microscopy.
35. Do not dry the surface of the chip, and do not let it with little media for a long time. The surface should not dry, because this increases the risk of introducing air bubbles into the microfluidic chip when injecting, and of dry media debris entering and damaging the microfluidic chip.
36. We find that it is better to not put all target suspension in the eppendorf at once, but rather add some more for each microfluidic experiment during the day. Indeed, in case of a problem with the pressure controller, one could lose the full content of the eppendorf. In addition, this allows the use of a suspension from the same fresh and clean stock for each acquisition.
37. With our design, our pressure controller, and when putting the reservoir on the side of the microscope stage at a height close to the one of the microfluidic chip, we find that setting the pressure around 12 mbar until liquid gets out of the injector, lowering the pressure to 8-9 mbar while inserting the metal injector into the hole, then injecting targets/lymphocytes with a pressure around 20 mbars, and setting to a resting pressure (very low flow to prevent arrival of more targets/lymphocytes) around 6-8 mbars (this can vary a lot depending on the design and the length of the tube) is a good starting point.
38. A good rule of thumb is that if the microscope settings allow to image 30 traps, then having 10-15 traps with a target/lymphocyte in it is enough (keeping in mind the field of view of the microscope). One should keep in mind that more targets/lymphocytes will

certainly arrive and be trapped in the next few minutes, during the wash with cell culture media, and that if there are traps containing several targets/lymphocytes, analysis will be compromised.

39. This manipulation is critical and will be repeated in the next step. It requires some training before being able to avoid injecting air bubbles, which can be mostly prevented by ensuring that the incoming metal injector has media going out of it. Another risk is to trigger backflow and have targets flowing back to the entry.
40. We find that taping it to the ceiling of the 37°C box of the microscope is most convenient.
41. As an example of imaging settings, with a spinning disk confocal microscope, we could do 40 minutes acquisitions with 1 image every 30 s, at 3 positions, in 3 colors (Transmission + 2 fluorescent channels), and 21 z slices, using a spinning disk confocal microscope and streaming each channel. For the cell line microfluidic chip design, the Nikon perfect focus system does not work, but one can use the software autofocus on the transmission channel at each time point, and an offset on the z-position for the fluorescent channels.

5. References

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Figure captions

Figure 1: (a) Bright field image of one of the chambers of the microfluidic chip designed for lymphocyte cell lines. Each microfluidic chip has four chambers in parallel, each containing a large array of microfluidic traps. Scale bar 100 μm . (b) (Left) Microfluidic traps designed for the B lymphocyte cell line (diameter $\sim 14 \mu\text{m}$) and the (Right) design adapted to primary lymphocytes (diameter $\sim 7 \mu\text{m}$). Scale bar 10 μm .

Figure 2: Functionalization of oil droplets. The stock emulsion is prepared as described in section 3.3 Droplet formulation. Oil droplets presenting biotinylated phospholipids at their surface are sequentially functionalized with a fluorescent streptavidin and a biotinylated ligand, here a biotinylated BCR ligand acting as antigen. Images of diluted, functionalized droplet suspension by spinning disk confocal microscopy below. Scale bar 20 μm .

Figure 3: Steps for immune synapse reconstitution in the microfluidic chip.

Figure 4: Examples of images obtained using this system, on a spinning disk confocal microscope. (a) B lymphocyte cell line (IIA1.6) stained with Hoechst to visualize the nucleus, in contact with an antigen-coated droplet. (b) Naive primary murine B lymphocyte expressing LifeAct-GFP, stained with Hoechst to visualize the nucleus, in contact with an antigen-coated droplet. Illustrations are z-projections over a few planes at the center of the cell, from z-stacks covering the full depth of the cell. Scale bar 5 μm .

Figures

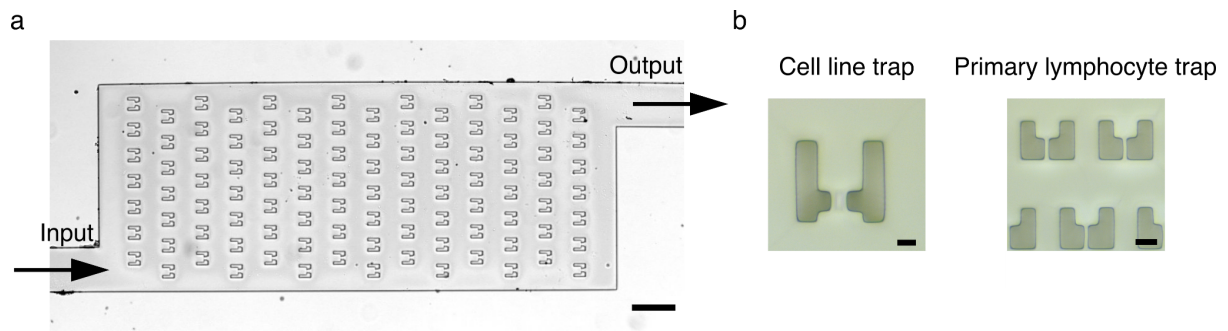


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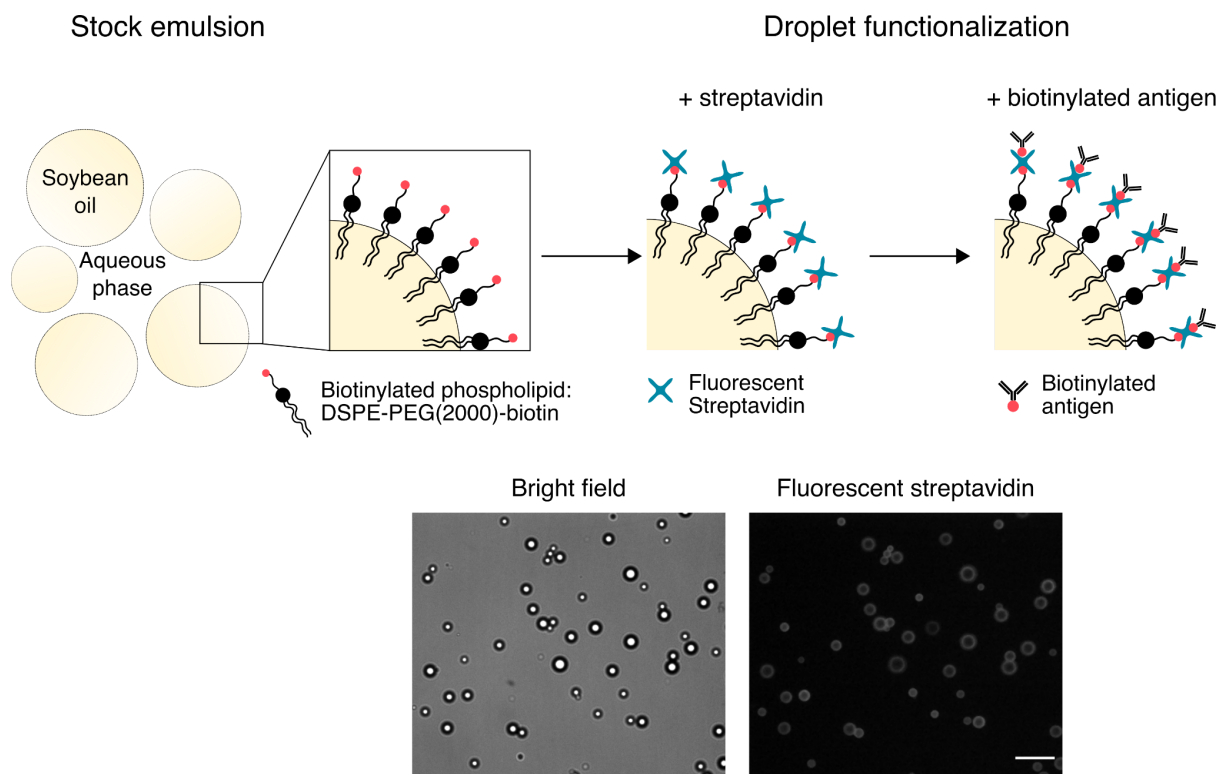


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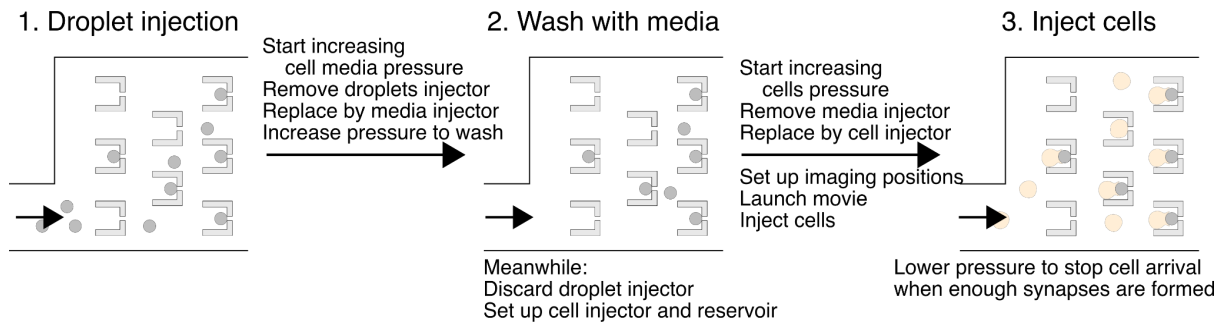


Figure 3: Steps for immune synapse reconstitution in the microfluidic chip.

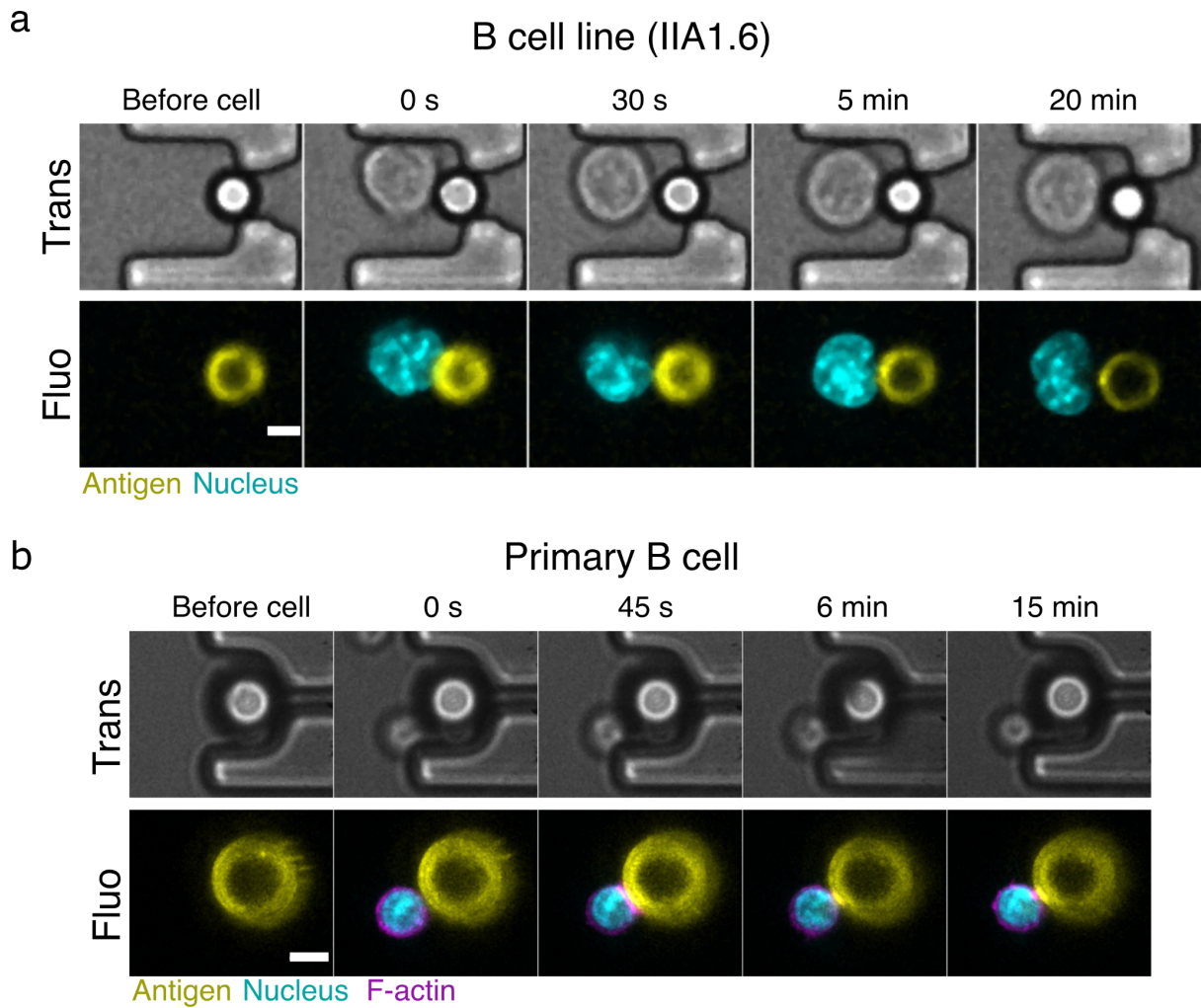


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