Video Article

Light-sheet Microscopy for Three-dimensional Visualization of Human Immune Cells

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Abstract

In vivo, activation, proliferation, and function of immune cells all occur in a three-dimensional (3D) environment, for instance in lymph nodes or tissues. Up to date, most *in vitro* systems rely on two-dimensional (2D) surfaces, such as cell-culture plates or coverslips. To optimally mimic physiological conditions *in vitro*, we utilize a simple 3D collagen matrix. Collagen is one of the major components of extracellular matrix (ECM) and has been widely used to constitute 3D matrices. For 3D imaging, the recently developed light-sheet microscopy technology (also referred to as single plane illumination microscopy) is featured with high acquisition speed, large penetration depth, low bleaching, and photocytotoxicity. Furthermore, light-sheet microscopy is particularly advantageous for long-term measurement. Here we describe an optimized protocol how to set up and handle human immune cells, e.g. primary human cytotoxic T lymphocytes (CTL) and natural killer (NK) cells in the 3D collagen matrix for usage with the light-sheet microscopy for live cell imaging and fixed samples. The procedure for image acquisition and analysis of cell migration are presented. A particular focus is given to highlight critical steps and factors for sample preparation and data analysis. This protocol can be employed for other types of suspension cells in a 3D collagen matrix and is not limited to immune cells.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57651/>

Introduction

Most knowledge about migrating cells comes from 2D experiments^{1,2,3}, which are normally conducted in a glass or plastic surface of a culture/imaging dish. However, a physiological scenario requires, in most cases, a 3D microenvironment, in which the extracellular matrix (ECM) plays a decisive role. ECM not only provides the 3D structure essential to maintain proper cell morphology but also offers survival signals or directional cues for an optimal functioning of many cells^{4,5}. Therefore, a 3D environment is required to better identify cellular functions and behavior in an environment better reflecting the physiological context.

In the human body, most cells especially immune cells, exert their functions under a 3D scenario. For example, activated T cells patrol tissues searching for target cells, naïve T cells migrate through lymph nodes in search for their cognate antigen-presenting cells during which the migration mode and machinery are adapted to the corresponding extracellular environment^{3,6,7}. The 3D collagen gel has been widely used as a well-established and well-characterized 3D cell culture system^{8,9,10}. Our previous work shows that primary human lymphocytes are highly mobile and migrate at an average speed of around 4.8 $\mu\text{m}/\text{min}$ in a 0.25 % collagen-based matrix¹¹. Rearrangement of cytoskeleton plays a key role in the cell migration¹². Accumulating evidence shows that lymphocytes do not apply only a single mode of migration yet can switch between certain migration behavior depending on the location, microenvironment, cytokines, chemotactic gradients, and extracellular signals which tune the migratory behavior in different ways³.

To reliably analyze immune cell functions and behavior, for example, migration, protrusion formation or vesicular transportation, it is of great advantage to be able to acquire images in relatively large 3D volumes in a fast and reliable manner. For 3D imaging, the recently developed light-sheet microscopy technology (also referred to as single plane illumination microscopy) offers a satisfactory solution^{13,14}. During imaging acquisition, a thin static light sheet is generated to illuminate the sample. In this way, on the focus plane, a large area can be illuminated simultaneously without affecting the off-plane cells. This feature enables a high acquisition speed with a drastically reduced bleaching and photocytotoxicity. In this paper, we describe how to visualize primary human immune cells using light-sheet microscopy and how to analyze the migration in a 3D scenario.

Protocol

Research carried out for this study with the human material (leukocyte reduction system chambers from human blood donors) is authorized by the local ethics committee (declaration from 16.4.2015 (84/15; Prof. Dr. Rettig-Stürmer)) and follows the corresponding guidelines.

1. Preparation of Neutralized Collagen Solution (500 μ L)

1. Transfer 400 μ L of chilled collagen stock solution (10.4 mg/mL) to a sterile 1.5 mL tube under the cell culture hood. Slowly add 50 μ L of chilled 10x PBS (pH 7.0 - 7.3) to 400 μ L of chilled collagen stock solution. Mix the solution by gently tilting the tube.
Note: All steps in Part 1 should be done under a cell culture hood.
2. Add 8 μ L of 0.1 M NaOH into 500 μ L of the collagen solution from 1.1. to adjust the pH to 7.2-7.6. Use pH test strips (pH range: 6 - 10) to determine the pH value of the mixture.
Note: Volumes may vary for different batches of collagen. NaOH solution has to be mixed slowly to avoid air bubbles. The mixture should be kept on ice to avoid collagen gelation.
3. Add 2 μ L of sterile ddH₂O to make the final volume to 500 μ L. Mix well and store this collagen solution (8.32 mg/mL) on ice or at 4 °C until further use.
Note: Under this condition, neutralized collagen can be used for 24 h. Aliquots are not recommended to avoid air bubbles.

2. Sample Preparation for Light-sheet Fluorescence Microscopy Using Capillaries

1. Fluorescently label the live cells of interest with the desired primary fluorescent dyes¹⁵ or fluorescent proteins¹¹ as described previously.
2. Transfer 1×10^6 of cells into a sterile 1.5 mL tube under a cell culture hood. Centrifuge the tube at 200 x g for 8 min. Discard the supernatant and resuspend the pellet in 200 μ L of culture medium.
Note: The cell density of 5×10^6 cells/mL is recommended for visualization of human immune cell migration, especially for cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.
3. Add 85.9 μ L of neutralized collagen solution from 1.3. into the cell suspension from 2.2. and mix properly to reach a collagen concentration of 2.5 mg/mL. Leave the cell/collagen mix on ice in the hood.
Note: Suppose the desired concentration of collagen is N mg/mL, the volume of neutralized collagen solution (for 200 μ L cell suspension) = $200 \times N / (8.32 - N)$
4. Next, put the matching plunger into the capillary (inner diameter ~ 1 mm) until the plunger is 1 mm out of the capillary. Wet the plunger by dipping into culture medium (**Figure 1A**).
Note: This step could help to prevent air bubbles when the capillary is dipped into the cell/collagen mix. The capillary and the plunger do not have to be sterile.
5. Dip the capillary into the cell/collagen mix from 2.3. Slowly pull the plunger back for 10 - 20 mm (**Figure 1B**). Wipe the outer wall of the capillary with a paper towel moistened with 70% ethanol spray to remove the remaining collagen solution.
6. Mount the capillary with modeling clay on the inner wall of a 5 mL tube and push the cell/collagen mix to the edge of the capillary (**Figure 1C**).
7. Keep the capillary at 37 °C with 5% CO₂ for 1 h for collagen polymerization.
8. Add the culture medium (around 1 - 2 mL) to a 5 mL tube. Carefully press the polymerized collagen rod out into the medium to around 3/4 of the collagen hanging in the medium (**Figure 1D**).
9. Keep the capillary, like this, at 37 °C with 5% CO₂ for another 30 min to equilibrate the collagen rod with the medium.
Note: Afterwards, the collagen rod can be pulled back into the capillary and cultured further before further use.

3. Image Acquisition using Light-sheet microscopy

1. Assemble the sample chamber according to the manufacturer's instruction.
2. Turn on the incubation and the microscope to heat the sample chamber to 37 °C (for live cell imaging only).
3. Place the capillary in the sample chamber and locate the sample to find the area of interest for image acquisition.
4. Activate the corresponding laser(s). Set the following settings: laser power, exposure time, step-size of z-stack, start and end position of z-stack, and the time interval for live cell imaging.
Note: For example, the sample in **Figure 2** was imaged every 40 s for 6 h at 37 °C with a step-size of 1 μ m (total thickness: 538 μ m). The laser power was 1% with an exposure time of 30 ms. The pixel size at x-y direction is 0.23 μ m.
5. Start the image acquisition.

4. Automated Tracking Analysis

1. Open the file converter, click **Add Files** to choose the imaging file(s) to be converted into the software file format (*.ims). Click **Browse** and select a folder to save the converted file(s). Click **Start All**.
2. Open the analysis software. Click **Surpass**. Go to **File** and click **Open**, then choose the imaging file to be analyzed.
Note: If the file size is big this step may take time. Files larger than 1 Terabyte (TB) are not recommended for a single experiment as the process such big files are highly computational capacity demanding.
3. Click **Add New Spots**. Check the box **Process Entire Image Finally**. Click **Next**.
4. Enter the coordinates for x and y (in pixel) to define the region of interest. Enter the number of frames (time and z-position) to analyze. Click **Next**.
5. Select the target channel, which contains the objects to be tracked, in the Dropdown list of **Source Channel**. Enter **Estimated xy Diameter** (in μ m). Click **Next**.
Note: Estimated xy diameter is the average diameter in the x-y dimension of objects to be tracked.
6. Click **Quality**. Set a threshold, in which most of the cells (objects) should be included. Click **Next**.
7. Choose the desired algorithm (**Autoregressive Motion** is recommended). Enter the max distance (**20 μ m** is recommended) and the max gap size (3 or 2 is recommended). Click **Process Entire Image finally**.
Note: **Max distance** and **Max gap size** are two thresholds to break tracks. More specifically, in two continuous frames when the distance between the same object exceeds the Max distance, this object in the later frame will be considered as a new object. Sometimes during

acquisition, the same object may disappear for a few frames and show up again. In this case, only when this object reappears within the Max gap size, it will be considered as the same object.

8. Click **Filter Type** and choose the option to exclude undesired tracks.
Note: This step is optional.
9. Click **Next** and then click **Finish**.
Note: This step can take hours up to days depending on computing performance.
10. Click **Edit Tracks** and choose **Correct Drift**. Select the appropriate algorithm (**Translational Drift** is recommended). Select the desired **Result Dataset Size** (**New Size Equal to Current Size** is recommended). Click **OK**.
Note: This step is only required when the collagen drifted during image acquisition.
11. Click **Statistics** and choose **Configure List of Visible Statistics Values**. Check the options of interest to be exported (e.g. coordinates, speed and so on). Click **OK**.
12. Click **Export All Statistics to File** and enter the file name.

5. Fixation and Immunofluorescence Staining of Cells in Collagen Matrices

1. Transfer 1,000 μL of 4% paraformaldehyde (PFA, in PBS) into a 5 mL tube under a chemical hood.
Note: PFA should be balanced to room temperature.
2. Dip the capillary with polymerized collagen from 2.9. into PFA solution (for ~ 5 mm) and mount the capillary on the inner wall of the 5 mL tube with modeling clay (as shown in **Figure 1C**).
3. Press the plunger gently until half of the collagen rod is hanging in the PFA solution (**Figure 1D**). Keep the tube at room temperature for 20 min.
4. Pull back the plunger to get the collagen rod inside the capillary. Take out the capillary and discard PFA.
5. Mount the capillary into a fresh tube and add 1 mL of PBS. Make sure that the capillary is immersed in the PBS.
6. Press the plunger gently until half of the collagen rod is hanging in the solution. Mount the capillary on the inner wall with modeling clay.
7. Keep the tube at room temperature for 5 min.
8. Repeat 5.4. - 5.7 for another 2 times.
9. Pull back the plunger to get the collagen rod inside the capillary. Discard PBS. Transfer 1 - 2 mL of blocking/permeabilization buffer (PBS + 1% BSA + 0.1% non-ionic surfactant) into the tube and repeat 5.6.
Note: The BSA (1%) can be replaced by 5% serum of the animal the secondary Ab was raised in.
10. Keep the tube at room temperature for 30 - 60 min.
11. Pull back the plunger to get the collagen rod inside the capillary. Discard the permeabilization buffer. Transfer 200 - 500 μL of the primary antibody in blocking/permeabilization buffer and expels the rod into the solution.
12. Keep the tube at room temperature for 1 h.
13. Wash the collagen rod 3 times with PBST (PBS + 0.1% non-ionic surfactant) as described in 5.4. - 5.8.
14. Incubate the rod in secondary antibody in blocking/permeabilization buffer for 1 h at room temperature. Keep it from light.
15. Wash the collagen rod 3 times with PBS as described in 5.4. - 5.8.
16. Pull back the plunger to get the collagen rod inside the capillary. Keep the samples in PBS until imaging.
17. Scan the samples as described in 3.

Representative Results

Protrusion formation during T cell migration is a highly dynamic process, which is actin dependent. To visualize protrusion formation of primary human CTL, we transiently transfected a mEGFP fused protein to label the actin cytoskeleton in CTL as described before¹¹. One day after transfection, the cells were embedded in the collagen matrix. Image stacks were acquired every 40 s with a step-size of 1 μm at 37 $^{\circ}\text{C}$ using light-sheet microscopy. As shown in **Figure 2A** and **Supplementary Movie 1**, during migration, human CTL form lemon-shaped major protrusions, fringed by fine spindle-like structures. In the experiment shown here, not only one single cell was imaged but a large volume ($450 \times 450 \times 538 \mu\text{m}^3$) (**Figure 2B**). Thus, the optical quality and resolution shown here were obtained with a low magnification objective (20X / 1.0 DIC VIS IR). Therefore, the resolution could be further improved with higher NA objectives.

In the experiment shown in **Figure 2** and **Movie 1**, CTLs were automatically tracked as described in the protocol Part 4. The trajectories of CTL are depicted in **Figure 2B**. Two parameters of migration were analyzed: velocity and persistence. Persistence is defined as the displacement divided by the total track length. The analysis shows that the mobility of CTL is very diverse in 3D: the velocities range from 0.01-0.19 $\mu\text{m/s}$ with an almost 20-fold difference, and the persistence ranges from 0 - 0.7 (**Figure 2C**). It was reported in mice that *in vivo* interstitial migration average velocity of T cells was 4 $\mu\text{m/min}$ ¹⁶. Recently, we have determined the average velocity of CTL to be 4 - 5 $\mu\text{m/min}$ ¹¹ using the methods described in this paper. These results demonstrate that light-sheet microscopy is a powerful tool to visualize cell behavior, for example, cell migration and cell-cell interaction. *In vivo*, many factors and a non-homogeneous ECM of the complex composition including soluble factors that can affect migration will modify T cell behavior.

Application of a collagen matrix is not limited to live cells. Fixation and immunostaining can also be performed in the matrix. **Figure 3** shows an example of fixed samples in 3D collagen gel, in which endogenous perforin1 (PFN1) and actin were stained. The sample was illuminated either from a single side (Single side illumination) or from both sides (Dual side illumination). We observe that cells at different z-positions are evenly stained, indicating that procedures described in our protocol achieve satisfactory penetration of antibodies into collagen gels. More importantly, after fixation CTL exhibited the same morphology as in live cell imaging (compare **Figure 3** and **Figure 2B**), indicating that also the morphology is well maintained by this protocol. In addition, illumination from the single side or from both sides does not make a significant difference in the quality of images at the focal plane. Considering that less area is exposed to the laser with the mode of single side illumination compared to dual side illumination, single side illumination is more recommended.

Figure 1: Illustration of handling capillaries during sample preparation. (A) Put the plunger into the capillary and how to wet the plunger with the culture medium. (B) Pull the cell/collagen mix into the capillary. (C) Mount capillaries using modeling clay. The plunger and the collagen rod are depicted in gray and red boxes, respectively. (D) Handle collagen rod for equilibration with the culture medium or for immunostaining. The plunger and the collagen rod are depicted in gray and red boxes, respectively. [Please click here to view a larger version of this figure.](#)

Figure 2: Tracking primary human CTL in 3D collagen and visualizing actin dynamics. (A) Maximum intensity projection of actin in primary human CTL. Actin was labeled in CTL as previously described¹¹. Day one post transfection cells were embedded in 0.5 % collagen matrix. One representative cell is shown. Scale bars are 5 μm . Fine actin structures at the protrusion edge are highlighted by the arrowhead. (B) Trajectories of CTL for 6 h. The trajectories are automatically tracked by the software (Color code: blue = start, red = end of the track, $450 \times 450 \times 538 \mu\text{m}^3$ volume). The scale bar is 50 μm . (C) Quantification of velocity and persistence. One dot represents one cell. Results are shown as mean $\pm$ SEM from 4 donors. [Please click here to view a larger version of this figure.](#)

Figure 3: Immunostaining of endogenous profilin1 (PFN1) and actin in CD8⁺ T cells embedded in the 3D collagen matrix. Primary human CD8⁺ T cells were embedded in 0.25% collagen. After PFA fixation the sample was stained using anti-PFN1 (orange) and phalloidin (purple) and visualized by light-sheet microscopy at a step-size of 1 μm for a total volume of $440 \times 440 \times 1,000 \mu\text{m}^3$. The sample was illuminated either from a single side (Single side illumination) or from both sides (Dual side illumination). Maximum intensity projections (MIP) from 3D reconstruction are shown. The scale bars are 50 μm . [Please click here to view a larger version of this figure.](#)

Movie 1: Generation of protrusions during T cell migration. The cell shown in the movie was taken from the volume shown in **Figure 2A**. Cells were embedded in a collagen matrix (0.5%). Images were acquired every 40 s for 6 h at 37 °C using light-sheet microscopy. [Please click here to view this video.](#) (Right-click to download.)

Discussion

Most *in vitro* assays are carried out on a 2D surface, for example in cell-culture plates, Petri-dishes or on coverslips, whereas *in vivo* cells, especially immune cells, experience mostly a 3D microenvironment. Emerging evidence shows that migration patterns of immune cells differ between 2D and 3D scenarios¹⁷. Moreover, the expression profiles of tumor cells are also different in 2D- and 3D-cultured tissues^{18,19,20}. Therefore, to best simulate the physiological conditions *in vitro*, it is of a great advantage to perform experiments in a 3D context, for instance in 3D collagen matrices. The newly developed approaches, in particular, the light-sheet fluorescence microscopy, enables reliable and reproducible experiments in 3D matrix systems under controlled environmental conditions.

In contrast to a conventional laser-scanning confocal microscopy which illuminates the whole probe, with light-sheet microscopy, only a thin sheet of laser light is generated to illuminate samples. The detection camera is perpendicular to the illuminating plane. Owing to this technique, only the section at the focal plane is exposed to the laser. Therefore, photo-bleaching and phototoxicity can be minimized. In addition, compared to point-scanning microscopy, the acquisition rate is drastically enhanced with light-sheet microscopy (100 - 1,000-fold faster) and the signal-to-noise ratio is also ameliorated. Combined with the incubation chamber of light-sheet microscopy setup used in this paper this microscope is a powerful option for image acquisition of large 3D volumes with minimized bleaching and photo-damage over extended periods of time (several days) under incubator conditions.

For sample preparation with the 3D collagen matrix, the most critical point is to avoid air bubbles. Given its high viscosity, when air bubbles are introduced to the collagen solution it is very difficult to remove it from the solution. The presence of air bubbles can induce heterogeneity of the polymerization of collagen matrix; in addition, it can block the path of light, which would diminish the quality of images. Therefore, when transfer or mix the collagen solution one must pipette very gently and do not expel the last drop in the pipette tip. Moreover, collagen starts to slowly polymerize at room temperature. Thus, to prevent polymerization before mixing the solution well, the collagen-containing tubes should be always kept on ice.

One big challenge we encounter in employing light-sheet microscopy is data handling. The data generated by light-sheet microscopy can easily reach the size of 500 gigabytes or even several terabytes, in particular for long-term experiments. To analyze these data takes several days, sometimes up to a week by a work-station with high calculation capacity. Since the size of analyzed images is not smaller than the original data the capacity of storage can quickly become a limiting factor for the application of light-sheet microscopy. Therefore, for tracking experiments or if complex image analysis is needed it is highly recommended to limit the file size per experiment to values that can be handled in a reasonable time frame by the available processing units.

Light-sheet fluorescence microscopy introduced in here has some features, which may affect the results if not handled carefully. The utilization of capillaries is particularly advantageous for a barrier-free illumination of the samples. Since the collagen rod is hanging in the medium, it may happen that the collagen rod drifts during image acquisition, especially for long-term measurements. This drift has to be corrected to avoid misinterpretation of the results. Of course, there are other light-sheet microscopy setups commercially available on the market, which do not require capillaries to mount the samples. Furthermore, with the light-sheet microscopy, besides the fact that compared to the conventional line scanning confocal microscopy phototoxicity has been substantially reduced, phototoxicity still remains a concern for long-term imaging²¹.

Apart from light-sheet microscopy, there are other approaches established for the 3D fluorescence imaging, among which the mostly applied are confocal microscopy, wide-field fluorescence microscopy, and multiphoton microscopy. In terms of penetration depth, confocal and wide-field fluorescence microscopy lie in the similar range, normally limited to 100 nm²². Owing to the longer wavelength light utilized for multiphoton microscopy, its penetration depth can be enhanced to the range of 0.5 - 1 mm²³. In comparison, the size of multi-dimensional images generated by optical sectioning of light-sheet microscopy can be up to several millimeters²⁴. In terms of lateral resolution, an elegantly performed experiment shows that light-sheet microscopy provides a better three-dimensional spatial resolution compared to confocal fluorescence microscopy in large samples²⁵. Recently, light-sheet microscopy has been combined with two-photon technique, which further improves the penetration depth compared to normal one photon light-sheet microscopy and is ten times faster than point-scanning two-photon microscopy²⁶.

Considering 3D matrix that provides the micro-architecture for cells, besides bovine collagen I used in this paper, there are many other options, such as extra-cellular matrix (ECM) derived from mouse sarcoma cells, agarose, synthetic hydrogels or other biocompatible polymeric materials. Mouse sarcoma ECM is a protein mixture including collagen IV, laminin as well as numerous growth factors. Compare to the synthetic materials, biological ECMs (collagen and mouse sarcoma ECM), on one hand, present a more physiologically relevant context; but on the other hand, the quality and composition biological ECMs may vary between batches and companies. In contrast, the reproducibility of synthetic materials is more guaranteed. Remarkably, fine-tuning of biochemical and mechanical properties, as well as desired modifications, is allowed for synthetic materials, but not for biological ECMs^{10,27}.

In summary, in this paper we described a protocol to construct 3D collagen matrix for cell biology experiments using light-sheet fluorescence microscopy, and how to embed primary human CTL in the 3D collagen matrix to visualize and analyze CTL migration. We highlighted the key points in sample preparation, image acquisition, and the analysis. Our results show that formation of highly dynamic protrusions and fine actin structures can be visualized by light-sheet microscopy with a good spatiotemporal resolution. Moreover, the analysis protocol described here enables us to reliably track cells in an objective and automated manner. This method will be especially advantageous for human immunological research as mice and humans differ substantially in many immunological functions and reactivity to therapies²⁸.

Disclosures

The authors declare no financial or commercial conflict of interest.

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