

Video Article

Revealing the Cytoskeletal Organization of Invasive Cancer Cells in 3D

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Abstract

Cell migration has traditionally been studied in 2D substrates. However, it has become increasingly evident that there is a need to study cell migration in more appropriate 3D environments, which better resemble the dimensionality of the physiological processes in question. Migratory cells can substantially differ in their morphology and mode of migration depending on whether they are moving on 2D or 3D substrates. Due to technical difficulties and incompatibilities with most standard protocols, structural and functional analysis of cells embedded within 3D matrices still remains uncommon. This article describes methods for preparation and imaging of 3D cancer cell cultures, either as single cells or spheroids. As an appropriate ECM substrate for cancer cell migration, we use nonpepsinized rat tail collagen I polymerized at room-temperature and fluorescently labeled to facilitate visualization using standard confocal microscopes. This work also includes a protocol for 3D immunofluorescent labeling of endogenous cell cytoskeleton. Using these protocols we hope to contribute to a better description of the molecular composition, localization, and functions of cellular structures in 3D.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50763/>

Introduction

The field of cell migration has been braving into the brand new third dimensional world. It is intuitive to study cell migration in an environment that most closely resembles the physiological one and, therefore, three-dimensional (3D). However, due to technical limitations, most cell migration studies have been done analyzing cell movement across rigid two-dimension (2D) substrates, either untreated or coated with appropriate extracellular matrix (ECM) proteins.

The first studies dedicated to cell migration in three dimensional collagen lattices go back over 20 years¹⁻³. However, only over the past 5 years it has become clear that migratory cells could substantially differ in their morphology and mode of migration depending on the dimensionality of the substrate. In 2D, cells only contact the substrate with their ventral surface using focal adhesions, resulting in the formation of broad flat protrusions (lamellipodia) with embedded finger-like protrusions (filopodia) at their leading edge. These structures, together with stress fibers that connect the cell front to the trailing edge, are believed to be crucial for cell movement in 2D. In 3D matrices, cells are generally more elongated, with the entire cell surface contacting the ECM, causing considerable changes in the formation and functional relevance of many of these structures. Conversely, other cellular features gain relevance in 3D migration, such as nuclear deformation and structures involved in ECM remodeling⁴.

Despite these known morphological alterations, as well as differences in migration modes⁵⁻⁷, which can vary depending on the ECM and cell types, structural and functional analysis of cells embedded within 3D matrices still remains unusual. Working with thick and dense 3D matrices carries technical difficulties, such as high-resolution microscopy imaging, and incompatibilities with most standard protocols optimized for 2D cultures, like immunofluorescent labeling of endogenous proteins. Also, because the use of 3D matrices is a relatively new approach, researchers are still investigating the best conditions to closely resemble specific *in vivo* situations, such as the normal stromal architecture of different tissue organs or the ECM organization around a tumor. Discrepancies in results by different groups concerning, for instance, cancer cell modes of migration or the existence of focal adhesions, have generated some controversy⁸. A lot of effort has been recently dedicated to reach a consensus in terms of ECM chemical nature, pore size, fiber thickness, and matrix stiffness. Many different types of 3D ECMs are currently used, varying from cell derived matrices to commercially available matrigel, pepsinized bovine collagen I, or nonpepsinized rat tail collagen I. Each of these matrices has specific physical and chemical properties and one needs to relate the matrix of choice to the physiological process being studied. In addition, pore size and fiber thickness can depend on polymerization conditions, such as pH and temperature^{9,10}. Binding to and distance from rigid substrates such as glass, can also change the elastic properties of the matrix^{10,11}.

This article describes methods for preparation and imaging of 3D cancer cell cultures, either as single cells or spheroids. Methods for making cancer cell spheroids have previously been described, the most popular ones being the hanging drop method^{12,13} and the agarose-coated plate method¹⁴. As an appropriate ECM substrate for cancer cell migration, nonpepsinized rat tail collagen I polymerized at room-temperature is used at 2 mg/ml. Nonpepsinized acid-extracted collagen I from rat tail retains both N- and C-terminal telopeptides, nonhelical portions of the collagen molecule responsible for native collagen intermolecular crosslinking and fibrillar stability¹⁵. Together, these conditions allow the formation

of collagen networks that most closely resemble the ones observed *in vivo*¹⁰. To allow visualization of the collagen fibers, both in fixed and living cultures, a detailed protocol is provided to fluorescently label collagen *in vitro*¹⁰ using 5-(and-6)-carboxytetramethylrhodamine (TAMRA), succinimidyl ester. This protocol has been adapted from Baici *et al.*^{16,17}, where fluorescein isothiocyanate is used to label soluble collagen molecules. As fluorescein, TAMRA is an amino-reactive fluorescent dye that reacts with nonprotonated aliphatic amino groups of proteins, such as the free amino group at the N-terminus and, more importantly, the side amino group of lysines. This reaction only occurs at basic pH, when the lysine amino group is in the nonprotonated form. In addition to TAMRA being more stable than fluorescein over time, its emission spectra falls on the orange/red range (ex/em = 555/518 nm), which can be usefully combined for live cell imaging of GFP-tagged proteins. Using soluble collagen labeled molecules with amino-reactive dyes does not affect the polymerization process nor the density, pore size and crosslinking status of the collagen matrix^{10,16,18,19}.

This protocol also includes a method for 3D immunofluorescent labeling of endogenous proteins, which has been further optimized to label the cytoskeleton or cytoskeleton associated proteins. The final focus of this protocol is on methods to acquire high-resolution images of 3D cultures using confocal microscopy with reduced contribution from rigid glass coverslips on the collagen matrix tension.

Protocol

1. TAMRA-collagen I Labeling

1. Prepare a 10 mg/ml TAMRA solution by adding 2.5 ml DMSO to the supplied 25 mg TAMRA powder. Dissolve it by vortexing until complete dissolution. Store at -20 °C and protect from light.
2. Prepare 2 L of Labeling Buffer (0.25 M NaHCO₃, 0.4 M NaCl). Adjust pH to 9.5 using 10 M solution of NaOH. Keep at 4 °C. From this point, all operations are carried out at 4 °C unless otherwise stated and fluorescent material is protected from light using aluminum foil.
3. Fill a 1 ml disposable syringe with 1 ml of highly concentrated rat tail collagen I solution. High concentration collagen solutions are typically provided at concentrations around 10 mg/ml and are very viscous. Be sure to manipulate it slowly to avoid formation of air bubbles.
4. Using a 21 G hypodermic needle, inject the collagen into a presoaked 3 ml dialysis cassette of 10,000 MWCO cut off. Use caution to avoid damaging the membrane with the needle. Remove all air from the dialysis cassette by pulling up the syringe piston. Dialyze it overnight against 1 L of Labeling Buffer.
5. Mix 100 µl of 10 mg/ml TAMRA solution with 900 µl of Labeling Buffer. Note: This should be done with both TAMRA solution and Labeling Buffer at room temperature since DMSO freezes at 4 °C. After mixing, bring the diluted TAMRA solution back to 4 °C.
6. Carefully remove the collagen from the dialysis cassette using a 2 ml disposable syringe with a 21 G hypodermic needle. Mix 1 ml of the dialyzed collagen solution with 1 ml of diluted TAMRA solution by pipetting.
7. Transfer the collagen/TAMRA mix into a 2 ml microcentrifuge tube and incubate overnight with rotation.
8. Transfer the 2 ml of TAMRA-labeled collagen into a presoaked 3 ml dialysis cassette and dialyze overnight against 1 L of Labeling Buffer to remove the excess of free dye.
9. To restore the TAMRA-labeled collagen to the collagen original solution, place the dialysis cassette into 1 L of 0.2% (v/v) acetic acid solution, pH 4, and dialyze overnight.
10. Measure the final volume of the TAMRA-labeled collagen and calculate its final concentration, considering the initial volume and concentration of the collagen solution used. Store at 4 °C.

2. 3D TAMRA-collagen Matrices with Embedded Single Cells

1. Calculate the volume of 2 mg/ml TAMRA-Collagen Mix necessary for the experiment. Always prepare 20% more to account for pipetting losses due to the collagen high viscosity.
2. Prepare a stock solution of 10x PBS and 1 N NaOH. Filter-sterilize and maintain at 4 °C. From this point, all operations are carried out on ice unless otherwise stated and under sterile conditions.
3. Mix 10x PBS and 1 N NaOH in appropriate volumes to achieve the desired collagen concentration and pH 7.4. For example, for a final volume of 1 ml of TAMRA-Collagen Mix at pH 7.4, combine 100 µl of 10x PBS with 5 µl of 1N NaOH. Mix well.
4. Add the appropriate volumes of both TAMRA-labeled collagen and unlabeled collagen in a 1:6 ratio to achieve a **final** total collagen concentration of 2 mg/ml. For example, for a final volume of 1 ml of 2 mg/ml TAMRA-Collagen Mix, add 90.48 µl of 3.68 mg/ml TAMRA-labeled collagen and 415.71 µl of 4.01 mg/ml of unlabeled collagen. Mix well and slowly by pipetting, avoiding formation of air bubbles.
5. Add cells suspended in the appropriate volume of chilled cell medium without FBS to the TAMRA-Collagen Mix to obtain a final cell density of 10⁵ cells/ml and a final collagen concentration of 2 mg/ml. For example, for 1 ml of 2 mg/ml TAMRA-Collagen Mix, add 388.81 µl of media containing 10⁵ cells. Mix well and slowly by pipetting, avoiding formation of air bubbles. Confirm pH by testing 10 µl of the mix on a pH test strip.
6. Pipette 100 µl drops of TAMRA-Collagen Mix onto glass bottom dishes and allow it to polymerize at room temperature for 30-45 min. 100 µl collagen drops have a typical diameter of 7 mm and are 2 mm high. When polymerized, the collagen turns into a white-ish gel. Note: Allowing the collagen to polymerize without closing the dish will avoid the formation of a water film around the collagen drop, reducing detachment from the glass. To increase adherence, glass dishes can be coated with poly-L-lysine at 100 µg/ml.
7. Carefully add sufficient culture medium to cover the collagen/cell drops. Avoid high fluxes of media or abrupt movements since collagen drops can easily detach. Keep at 37 °C in 10% CO₂ humidified air for enough time for cells to migrate in the matrix (typically 1-3 days).

3. 3D TAMRA-collagen Matrices with Embedded Cell Spheroids

1. Calculate the volume of 2 mg/ml TAMRA-Collagen Mix necessary for the experiment. Always prepare 20% more to account for pipetting losses due to the collagen high viscosity.
2. Prepare a stock solution of 10x PBS and 1 N NaOH. Filter-sterilize and maintain at 4 °C. From this point, all operations are carried out on ice unless otherwise stated and under sterile conditions.

3. Mix 10x PBS, 1 N NaOH and cell media without FBS in appropriate volumes to achieve the desired collagen concentration and pH 7.4. For example, for a final volume of 1 ml of TAMRA-Collagen Mix at pH 7.4, combine 100 μ l of 10x PBS, 5 μ l of 1 N NaOH and 388.81 μ l of media. Mix well.
4. Add the appropriate volumes of both TAMRA-labeled collagen and unlabeled collagen in a 1:6 ratio to achieve a **final** total collagen concentration of 2 mg/ml. For example, for a final volume of 1 ml of 2 mg/ml TAMRA-Collagen Mix, add 90.48 μ l of 3.68 mg/ml TAMRA-labeled collagen and 415.71 μ l of 4.01 mg/ml of unlabeled collagen. Mix well and slowly by pipetting, avoiding formation of air bubbles. Confirm pH by testing 10 μ l of the mix on a pH test strip.
5. Pipette 100 μ l drops of TAMRA-Collagen Mix onto glass bottom dishes and allow it to initiate polymerization for 2-5 min. This will help to prevent sinking of the spheroids by slightly increasing the gel viscosity. 100 μ l collagen drops have a typical diameter of 7 mm and are 2 mm high.
6. Collect a cell spheroid and place it on a clean Petri dish. Remove any excess of liquid and resuspend it in 10 μ l of TAMRA-Collagen Mix. This is important to prevent dilution of the collagen with cell media.
7. Using a P20 pipette, collect the collagen suspended spheroid and place it on the center top of the 100 μ l TAMRA-Collagen Mix drop. Note: Do not place more than one spheroid per collagen drop, since they can affect each other capacity to invade and/or migrate.
8. Allow the TAMRA-Collagen Mix to polymerize at room temperature for 30-45 min. When polymerized, the collagen turns into a white-ish gel. Note: Allowing the collagen to polymerize without closing the dish will avoid the formation of a water film around the collagen drop, reducing detachment from the glass. To increase adherence, glass dishes can be coated with poly-L-lysine at 100 μ g/ml.
9. Carefully add sufficient culture medium to cover the collagen/spheroids drops. Avoid high fluxes of media or abrupt movements since collagen drops can easily detach.
10. Keep at 37 °C in 10% CO₂ humidified air for enough time for cells to invade/migrate in the matrix (typically 1-3 days).

4. 3D Immunofluorescence Staining

1. Carefully remove the cell media and rinse the collagen matrices containing cells/spheroids with PBS.
2. Simultaneously fix and extract cells by incubating with extraction/fixation buffer (4% PFA, 0.3% Triton X-100, 5% sucrose in PBS) for 5 min. Supplement the buffer with 2 μ M Phalloidin and 2 μ M Taxol for visualization of cytoskeleton or cytoskeleton associated proteins.
3. Further fix cells with 4% PFA, 5% sucrose in PBS for 30 min. Rinse with 0.05% Tween-20 in PBS.
4. Prepare primary antibodies solutions in PBS. Incubate cells with primary antibodies for at least 1 hr at room temperature or overnight at 4 °C. Make sure to add enough solution to cover the entire collagen drop. For a 35 mm glass bottom dish, 1.5-2 ml of solution will be necessary. Note: To reduce the volume of antibody solution, a water insoluble barrier, such as a circle line of silicone grease or a PDMS ring, can be placed around the collagen drop.
5. Wash 3x 30 min with 0.05% Tween-20 in PBS.
6. Prepare Alexa-conjugated secondary antibodies, Alexa-conjugated phalloidin and DAPI solutions in PBS. Incubate cells with the appropriate secondary antibodies for 2 hr at room temperature.
7. Wash 3x 30 min with 0.05% Tween-20 in PBS.
8. Remove excess of liquid, add enough mounting media to fill the glass bottom dish bottom (approx. 500 μ l) and place a 24 mm coverslip on top to seal. Do not press down the coverslip to avoid compressing the collagen drop and damaging the 3D organization.

5. Sample Imaging

Classic or spinning disk confocal microscopes can be used. An inverted system should preferentially be used to determine the distance of the imaged cells from the glass bottom. The system used for this work is an Inverted Confocal Spinning Disk equipped with a 40X/1.3NA and a 60X/1.4NA oil-immersion objectives (working distances 200 μ m and 130 μ m, respectively), a Photometrics CoolSNAP HQ2 camera, 1392 x 1040 imaging array, 6.45 x 6.45 μ m pixels and controlled by Metamorph imaging software. 405 nm, 491 nm, 561 nm, and 633 nm lasers are typically used at 30–50% power, gain 1 and binning of 2 x 2 to reduce exposure times. The microscope is also equipped with a Hg lamp and filter cubes for DAPI (exc 400–418 nm; em 450–465), FITC (exc 478–495; em 510–555) and Texas Red (exc 560–580; em 600–650) to use with the eye piece.

1. Using the fluorescent lamp, place the 40X objective at the middle of the collagen drop. Get an overview of the sample, both in the x-y axis and in the z-axis. Make sure not to deviate more than 100 μ m from the gel edge on the x-y axis when acquiring the images to avoid edge effects. When imaging spheroids, make sure they are under the objective working distance. Change objective if appropriate.
2. Switch to the confocal live imaging mode. By using the 561 nm laser to visualize the TAMRA-labeled collagen, starting from the glass bottom determine the z value at which collagen fibers start to appear. This is the substrate bottom and z = 0.
3. Using the focus knob, go up 100 μ m from z = 0. Only cells at z = 100 μ m or above should be imaged to avoid tension effects from the rigid glass bottom.
4. Select the cells to image. Optimize camera exposure times and laser power for each wavelength. Typical exposure times for DAPI and Alexa-488 Phalloidin are between 100-200 msec. Exposure times for antibody staining may vary.
5. On confocal live mode using the appropriate laser to visualize the phalloidin staining and using the focus knob, define the z series interval by setting top and bottom z values to current.
6. Define the z-step size. For 40X objectives, use 1 μ m. For 60X, use 0.5 μ m. Start acquisition.

Representative Results

Labeling rat-tail collagen I with TAMRA allows an easy preparation and visualization of 3D collagen networks. Slow polymerization at room temperature results in the formation of collagen networks with comparable organization to the ones found *in vivo*¹⁰.

Here we present a protocol for immunofluorescence staining of the cytoskeleton of CT26 cancer cells, both as spheroids and as single cells. To better preserve the cytoskeleton, buffers are supplemented with unlabeled phalloidin and taxol, drugs known to stabilize F-actin and microtubules, respectively. In addition, cells are simultaneously fixed and extracted to remove nonpolymerized cytosolic tubulin pools that could

interfere with the visualization of individual microtubules. This technique can equally be used to visualize cytoskeleton-associated proteins. When in 3D, CT26 cells present a typical mesenchymal morphology characterized by an elongated cell body, tipped with F-actin rich cellular protrusions that resemble filopodia and lamellipodia (**Figures 1 and 2**). This elongated morphology is even more evident in cells attempting to escape cellular spheroids by invading the collagen matrix (**Figure 2**). Staining of microtubules using an anti-tubulin antibody shows a well-preserved and organized microtubule network (**Figures 1 and 2**). This cell shape greatly differs from the one of CT26 cells in 2D substrates, where they usually have several leading edges with big broad flat lamellipodia and well-defined filopodia¹⁰.

To process the acquired images in this work, Imaris was used. This software automatically converts the acquired big z-stacks into a 3D projection of easy navigation and visualization in all planes (x-y, x-z and y-z) and different angles (**Figures 1, 2 and 3**, orthogonal views). Using the "Crop 3D" function, unnecessary z planes above or below the region of interested can be removed. As represented in **Figure 3**, it is crucial to analyze collected z stacks from all planes of view to ensure a uniform 3D distribution of the cells. In this case, CT26 cells grown as spheroids seem to invade a 3D collagen matrix extensively when visualized as a maximal x-y projection. However, an x-z view reveals that all cells are invading a restricted z interval compared to the spheroid volume, suggesting a preferential region of invasion. This spheroid is in fact too close to the glass bottom of the dish and cells moved fast towards and on the rigid 2D substrate.

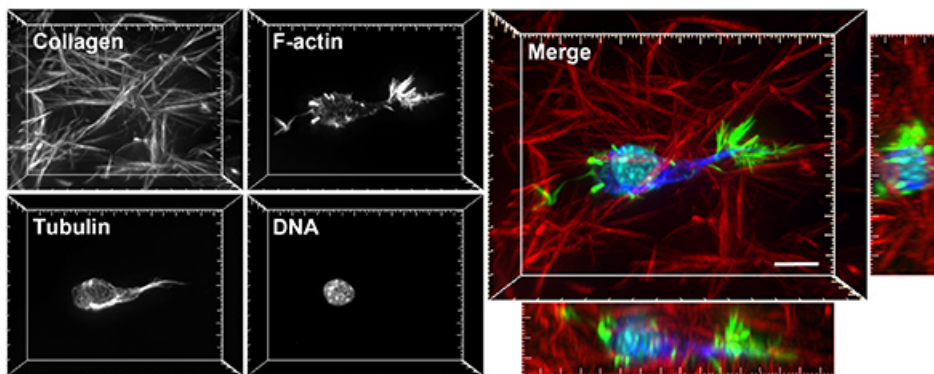


Figure 1. Cytoskeleton of CT26 cells cancer cells embedded in TAMRA-labeled collagen I. Colon adenocarcinoma CT26 cells were embedded as single cells in 2 mg/ml TAMRA-labeled collagen I (red). Immunofluorescence images of cultures stained with a tubulin antibody to label microtubules (blue), Alexa-488 phalloidin to visualize F-actin (green) and DAPI for nuclear staining (cyan). 3D image corresponds to x-y maximal projection of z-stacks of 38 µm. Orthogonal x-z (bottom of Merge) and y-z (right of Merge) merge views. Scale bar, 20 µm. [Click here to view larger image.](#)

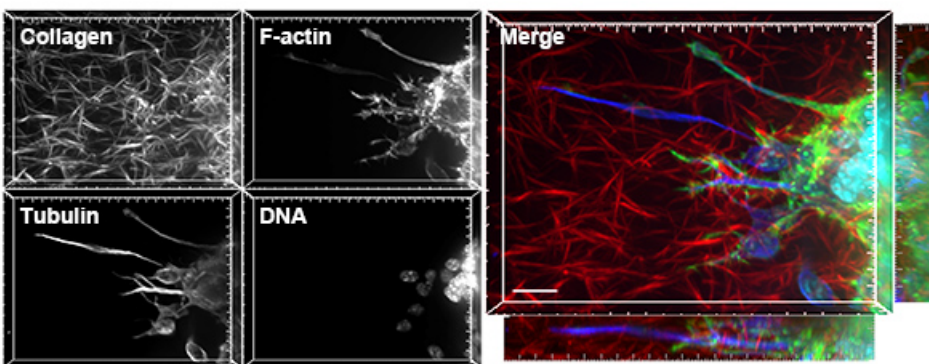


Figure 2. Cytoskeleton of CT26 cells cancer cells invading TAMRA-labeled collagen I. CT26 cells grown as cellular spheroids were embedded in 2 mg/ml TAMRA-labeled collagen I (red). After 2 days in culture, cells started invading the collagen 3D matrix, moving away from the cell spheroid. Immunofluorescence images of cultures stained with a tubulin antibody to label microtubules (blue), Alexa-488 phalloidin to visualize F-actin (green) and DAPI for nuclear staining (cyan). 3D image corresponds to x-y maximal projection of z-stacks of 66 µm. Orthogonal x-z (bottom of Merge) and y-z (right of Merge) merge views. Scale bar, 50 µm. [Click here to view larger image.](#)

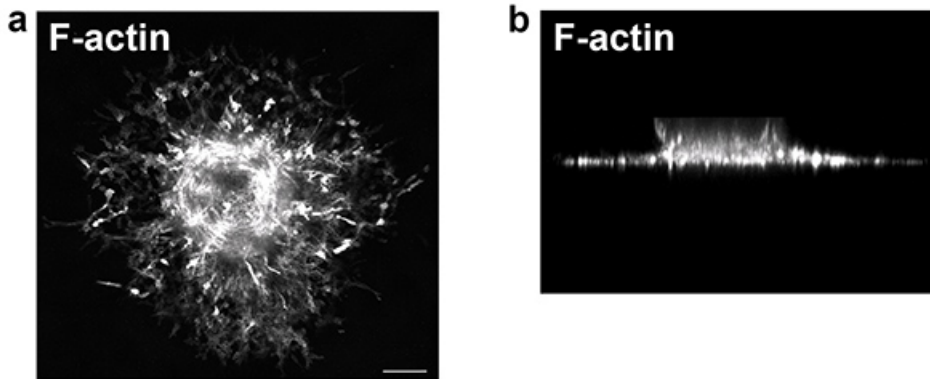


Figure 3. CT26 cells invasion in collagen I is dependent on the glass distance. CT26 cells were grown as cellular spheroids and embedded in 2 mg/ml collagen I. After 2 days in culture, cells significantly moved away from the cell spheroid, not by invading the 3D collagen matrix but by crawling on the 2D rigid glass. Fluorescence images of cultures stained with Alexa-488 phalloidin to visualize F-actin. **a)** 3D image corresponds to x-y maximal projection of z-stacks of 200 μm . **b)** Orthogonal x-z view. Scale bar, 150 μm . [Click here to view larger image.](#)

Discussion

The protocol described here to fluorescently label collagen I using TAMRA provides an excellent method to allow easy visualization of the collagen network organization, by using a standard confocal microscope equipped with a 561 nm laser. An advantage of this technique comparing to reflectance confocal microscopy is the ability to image collagen fibers deeper into the 3D matrix. The intensity and contrast of the fibers reflection diminishes substantially with depth due to laser light absorption and scattering. Also, the orientation of the collagen fibers can be a major handicap when using reflectance confocal microscopy, since fibers aligned around 50° from the imaging plane are entirely undetected. Fluorescently-labeled fibers are detected with similar brightness, independent of their orientation²⁰. Another method that has been increasingly used to visualize collagen bundles, both *in vitro* and *in vivo*, is by second harmonic generation (SHG). This process is based on the emission of SHG signal by noncentrosymmetric structures, such as collagen²¹. However, SHG requires a multiphoton microscope, which is not part of the standard lab imaging equipment.

The use of TAMRA as a fluorophore is not exclusive. Other fluorophores, such as Cy2, Cy5 or the Alexa Fluor family, commercially available in protein labeling kits, can be used to label collagen in the most convenient color for the user. Another advantage of using fluorescent collagen is the potential to be combined with cells transfected with fluorescently-tagged proteins for time-lapse imaging, to closely follow cell-matrix interactions or cell-induced matrix deformations. It can also be used for thin collagen coating coverslips in 2D experiments.

Embedding cells in 3D matrices is a delicate procedure, particularly when using big spheroids, since they have a tendency to sink due to gravity. Also, cells are usually attracted to stiffer environments and when embedded in regions of the 3D matrix close enough to the glass coverslip to feel the increase in tension induced by the glass, they move towards the rigid 2D substrate. A technical trick to help prevent sinking of big spheroids is to allow the collagen to briefly polymerize (2-5 min) before placing the spheroid on top of the matrix drop. This will slightly increase the viscosity of the gel, increasing the sinking time. Nevertheless, it is good practice to prepare several replicates to increase the likelihood of success. On the other hand, it is important to keep the cells under the objective working distance. Lower magnification objectives usually have bigger working distances and can be used for overall field images, for instance, to compare invasion of different cell types from a spheroid. When higher resolution images are required, water immersion objectives, with bigger working distances, are greatly recommended.

Most of the published work looking at intracellular proteins of cells in 3D matrices has been done by over-expression of fluorescently tagged proteins, possibly due to the lack of reliable immunofluorescence staining protocol compatible with the 3D nature of the substratum. Problems such as antibody accessibility to cells deeply embedded or high background staining of the matrix (since many antibodies can unspecifically decorate the collagen fibers) can be a reason. This protocol also describes immunostaining in 3D collagen matrices. Although this protocol has been optimized for cytoskeleton or cytoskeleton associated proteins, it has also been successfully used to label focal adhesion markers, such as paxillin, vinculin and zyxin, in different cell lines¹⁰. We do not attempt to create a general 3D immunostaining protocol. As in 2D, each antibody may require a different fixation procedure and design of a specific protocol for each antibody might be required. This protocol is presented as starting point, and it is suggested that users include a fluorescent phalloidin staining to provide an idea of the cell shape and localization within the matrix.

Thick collagen matrices are a good simplified *in vitro* system to study cell migration in a physiological ECM. Although it lacks the chemical and physical complexity of a living tissue, this system allows manipulation of specific ECM properties, such as pore size, elasticity and crosslinking. We hope these protocols will contribute to a better description of the molecular composition, localization and functions of cellular structures for many years analyzed in 2D and to improve our knowledge of cell behavior in 3D.

Disclosures

The authors declare that they have no competing financial interests.

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