BioResearch

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Immunofluorescence Visualization of Fibroblast or Cancer Cells in RAFT™ 3D Cell Cultures

Technical Notes

Introduction

In this technical note, we show that immunofluorescence can be performed easily and routinely on RAFT™ 3D Cell Cultures. In the examples below, primary human dermal fibroblasts (HDF) or the breast cancer cell line MCF7 were cultured in RAFT™ 3D culture system for up to 23 days prior to fixation and staining. Depending on cellular behaviour we provide here two protocols which differ slightly depending on whether the cells maintain themselves singly within the culture or whether, as in the case of a number of cancer cell lines, they come together to form higher order structures or aggregates which we have termed tumoroids.

Material

The following materials were used for making, fixing and staining the RAFT™ Cultures. HDF and MCF7 were used as an example of single cell cultures or cellular aggregates respectively, as indicated on the second line of the table below.

Single Cell Cultures	Cellular Aggregates
Primary human neonatal dermal fibroblasts	MCF7 cancer cell line (ATCC, USA)
Growth Medium to support dermal fibroblasts	Minimum essential medium (MEM), GlutaMAX™ (Life Technologies) supplemented with 9% fetal bovine serum (Sigma) and antibiotics/ antimycotics (Life Technologies)
RAFT™ Absorbers and reagent kits, RAFT™ Instructions downloadable from www.lonza.com/raft under 'product knowledge center'. For a list of recommended manufacturer cell culture plates (either 96-well black wall or 24-well plate not supplied with the kit), contact Lonza scientific support team	RAFT™ Absorbers and reagent kits, RAFT™ Instructions downloadable from www.lonza.com/raft under 'product knowledge center'. For a list of recommended manufacturer cell culture plates (either 96-well black wall or 24-well plate not supplied with the kit), contact Lonza scientific support team
Formaldehyde solution, diluted to 3.7% (v/v) in PBS	Formaldehyde solution, diluted to 3.7% (v/v) in PBS
1X PBS	1X PBS
0.1% Triton X-100	1% Triton X-100
Blocking buffer: 1% (w/v) Bovine serum albumin in PBS	Blocking buffer: 1% (w/v) Bovine serum albumin in PBS + 0.2% Triton X100
Rat Anti-Tubulin antibody [YOL1/34] - Microtubule Marker (Abcam)	Rat Anti-Tubulin antibody [YOL1/34] - Microtubule Marker (Abcam)
Secondary antibody Cy3-AffiniPure Goat Anti-Rat IgG (H+L) (min X Hu,Bov,Hrs Sr Prot), Stratech Scientific	Secondary antibody Cy3-AffiniPure Goat Anti-Rat IgG (H+L) (min X Hu,Bov,Hrs Sr Prot), Stratech Scientific
Alexa Fluor 488® phalloidin (Life Technologies)	Alexa Fluor 488® phalloidin (Life Technologies)
DAPI (4',6-diamidino-2-phenylindole, dihydrochloride, Life Technologies)	DAPI (4',6-diamidino-2-phenylindole, dihydrochloride, Life Technologies)
Quenching solution: 1/50 dilution of stock solution (50mM Tris-HCl pH 7.5 + 1M Glycine) in PBS	Quenching solution: 1/50 dilution of stock solution (50mM Tris-HCl pH 7.5 + 1M Glycine) in PBS
Widefield fluorescent microscope fitted with a Z focus drive (in the example shown here, an Olympus IX71 inverted microscope with 10x and 40x phase contrast objectives and a Prior Z-focus drive, directly coupled to the microscope, were used).	Widefield fluorescent microscope fitted with a Z focus drive (in the example shown here, an Olympus IX71 inverted microscope with 10x and 40x phase contrast objectives and a Prior Z-focus drive, directly coupled to the microscope, were used).

Methods

In this example, the 96-well plate RAFT™ Cultures were prepared as indicated in the instructions downloadable from www.lonza.com/raft and seeded with either 5,000 or 50,000 human dermal fibroblasts (HDF) per well or 10,000 MCF7 cells per well. The cultures were then incubated at 37°C, with 5% CO_2 for 11 days (HDF at starting density of 5,000 cells/well), 3 days (HDF at starting density of 50,000 cells/well) or 23 days (MCF7) prior to being fixed and stained.

On the day of assay, the following protocol was used:

1. Each RAFT™ Culture was washed 3 times over a 15 min period with 100 µL PBS
2. The PBS was replaced with 100 µL of 3.7% formaldehyde* solution to fix the cells and the plate incubated at room temperature for 30 minutes
3. The formaldehyde solution was replaced with 100 µL of quenching solution to quench the formaldehyde cross-linking and the plate incubated at room temperature for 10 minutes
4. The RAFT™ Cultures were washed as in point 1
5. The PBS was replaced with 100 µL of either 1% or 0.1% Triton X-100 solution (for the MCF7 or HDF RAFT™ Cultures respectively) to permeabilise the cells and the plate was incubated at room temperature for 4 minutes
6. The RAFT™ Cultures were washed as in point 1
7. The primary antibody was diluted in the appropriate blocking buffer (in the case of our batch of YoI 1/34, a dilution of 1/100 was optimal, which was the same dilution that was optimal for cells cultured in 2D) and 50 µL of this solution was added in each well
8. The plate was then incubated overnight at 4°C
9. The RAFT™ Cultures were washed as in point 1
10. The secondary antibody, phalloidin if used (the recommended 1/40 dilution was used) and DAPI were diluted in the appropriate blocking buffer and 50 µL of this solution was added in each well
11. The plate was then incubated at room temperature for 2.5 hours
12. The RAFT™ Cultures were washed as in point 1
13. The wells were imaged on a fluorescence widefield microscope. Confocal imaging or the use of a high content imaging device would also be possible
14. If necessary, the plate can be stored at 4°C, in the dark, before being imaged

***Note:** It is also possible to fix the cultures with 100 µL of ice-cold methanol if the antibody used requires this fixation method for the best results.

Results

After overnight incubation of the primary antibody and 2.5 hours exposure to the secondary antibody, the fluorescent signal was bright enough to allow image capture with 100-200 ms exposure times for the anti-tubulin antibody and the phalloidin.

As shown in Figure 1, using a simple type of image deconvolution, such as ImageJ 3D parallel spectral deconvolution (http://rsb.info.nih.gov/ij/plugins/), can help improve the sharpness of the signal and remove background noise from the cells surrounding the cell of interest (such as the blurry, out of focus cell observed above the fibroblast imaged in Figure 1).

However, with our anti-tubulin antibody, images taken with our widefield microscope and not subjected to deconvolution were defined enough to observe microtubules within the cells in the RAFT™ 3D Culture (Figure 2).

Actin fibres can also be seen clearly in cells embedded in the RAFT™ Collagen matrix (Figure 2).

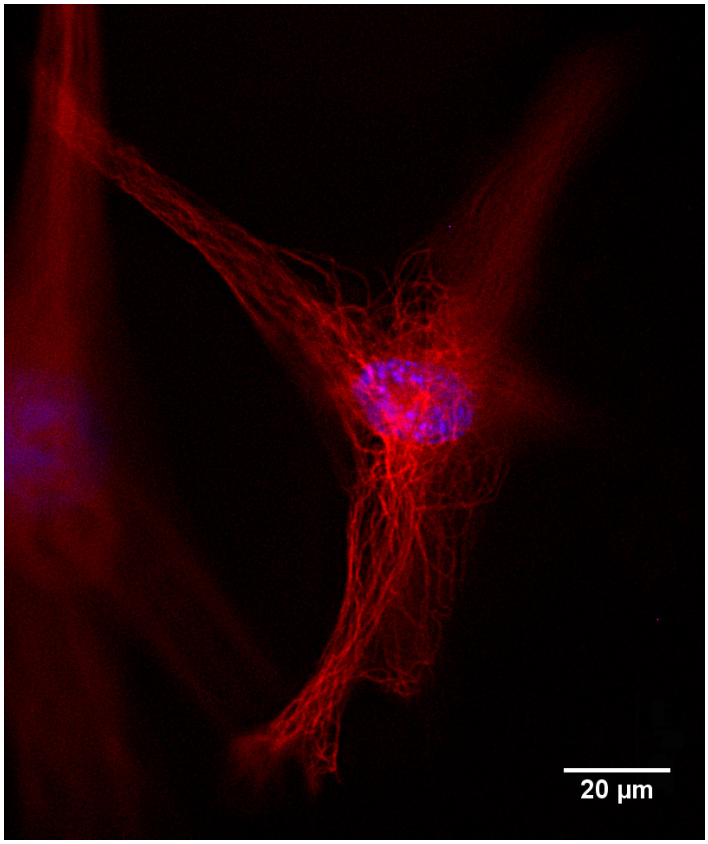

Figure 1: HDF cell fixed and stained for tubulin after being cultured in RAFT™ System for 11 days. One z-plane location was imaged here on a widefield microscope, after staining the RAFT™ Culture for tubulin (red) and the nucleus (blue). The image from each channel was deconvolved using the 3D spectral deconvolution software from ImageJ (generalised Tikhonov) and the merge of the two channels is shown here.

It has been found necessary when visualising structures within cellular aggregates such as tumoroids that a more intensive permeabilisation step is required to permit the access of the antibodies into the target proteins. With this small change to the protocol it is possible to capture detailed images for analysis (Figure 3). It should be noted that although the RAFT™ Process concentrates the collagen matrix to a physiologically relevant concentration the matrix itself does not act as a barrier to molecules as big as antibodies (150kDa), since no such permeabilisation steps were necessary when imaging single cells such as HDF in RAFT™ Cultures (Figure 1 and Figure 2).

After 3 days in culture, HDF have elongated within the collagen matrix and display a typical actin and microtubule cytoskeleton as can be seen in Figure 1 and Figure 2.
Each cell within the MCF7 tumoroids displayed a more rounded morphology (Figure 3).
As we can see in Figure 3, even after 23 days in culture, dividing cells can still be observed within the tumouroid.

Interestingly, the RAFT™ Collagen matrix is composed of rat tail collagen which, in this particular case, did not create a cross reactivity issue even when using an antibody, such as the anti-tubulin antibody, which was raised in rat. Moreover, the presence of the collagen matrix has little impact on the background fluorescence of the culture, in particular when the antibodies were diluted in a BSA-containing blocking buffer.

Conclusions

Cells cultured in the RAFT™ 3D Cell Culture collagen matrix can be fixed and stained using these standard immunofluorescence protocols. Depending on whether the cells of interest stay as single cells or aggregate into higher order structures such as cancer tumoroids the only modification required is to increase the permeabilisation of the structures to permit ingress of the antibodies. In addition the use of a blocking solution is advised to decrease the background from the collagen, however it has been possible to even use antibodies raised in rats.

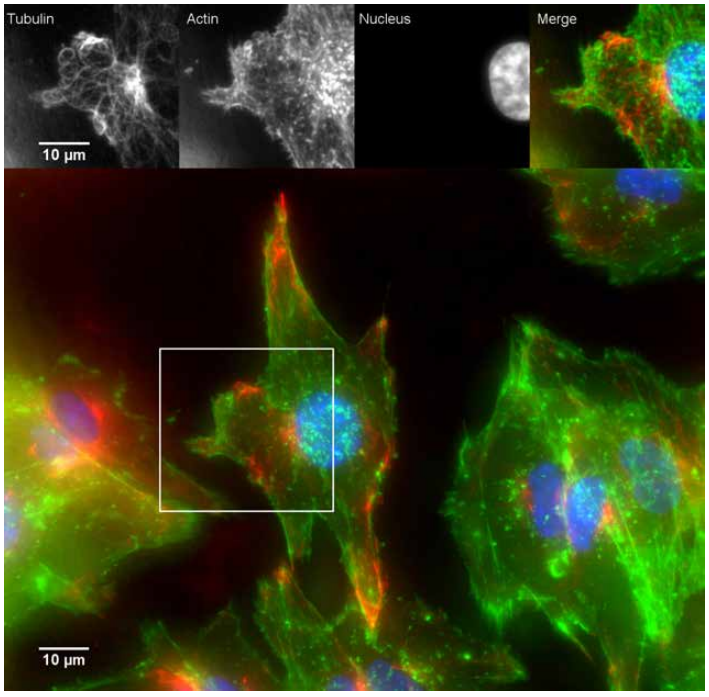

Figure 2: HDF cells fixed and stained for tubulin and actin after being cultured in RAFT™ System for 3 days. A series of z-planes taken at 0.5 µm intervals was imaged on a widefield microscope, after staining the RAFT™ Culture for tubulin (red), actin (green) and nucleus (blue). The z-stack from each channel was projected onto one plane using the maximum z-projection function of ImageJ software and the merge of all channels is shown in the large bottom panel. Across the top-panel one frame of the z-stack is shown, with each individual channel represented in grayscale separately, for the area boxed in the large bottom panel, to better show the detail of each staining.

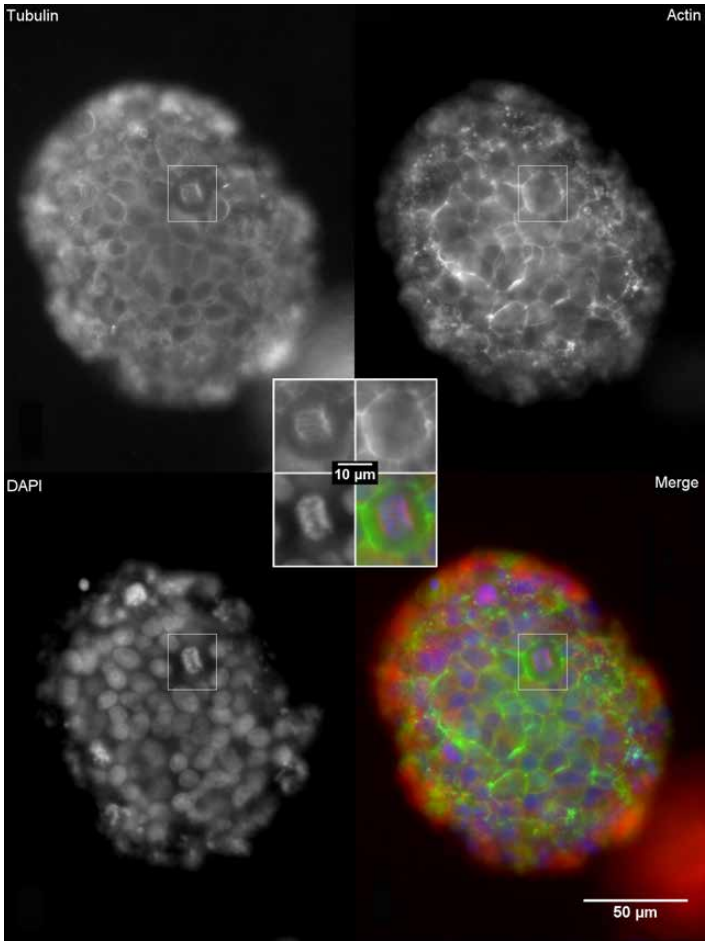

Figure 3: MCF7 tumoroids fixed and stained for tubulin and actin after being cultured in RAFT™ System for 23 days. One z-plane was imaged on a widefield microscope, after staining the RAFT™ Culture for tubulin (red), actin (green) and nucleus (blue). In the larger images around the exterior of Figure 3, each channel (labelled directly) is shown individually in grayscale, and then merged in the bottom right hand corner in colour. In the centre panels, a dividing cell indicated by a white box on the larger images is shown, for each channel and the merge, at a higher magnification.

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