**Live imaging of cell invasion using a multicellular spheroid model and light-sheet microscopy**

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**Running head**: live imaging of multicellular spheroids using light-sheet microscopy

**Summary/Abstract**

Three-dimensional cellular assays are becoming increasingly popular as a fundamental tool to bridge the gap between tissue culture systems and *in vivo* tissue. In particular, spheroids are recognised today as a necessary intermediate model between testing in monolayer cultures and testing in animals. This chapter describes a straightforward protocol, from sample preparation to image acquisition and initial post-processing, based on one of most widely used commercial light-sheet fluorescence microscopy platforms, the Zeiss Lightsheet Z.1.

**Key Words**

Multicellular Spheroid, Light-sheet Fluorescence Microscopy, Cell Invasion, FEP tubes, Matrigel, Hanging Drop, Glioblastoma, Multiview Reconstruction.

**1. Introduction**

The invasion of tumour cells into neighbouring tissue is a hallmark of cancer and constitutes the initial step of metastasis - the spread of tumour cells to distant tissues or organs (1). Metastasis contributes to over 90% of cancer deaths (2); however, the development of anti-metastatic therapies has been largely unsuccessful to date (3, 4), in part because of the absence of good predictive *in vitro* models for drug screening. 2D *in vitro* cell culture is currently the standard model used for initial preclinical drug testing. Yet, this model fails to replicate essential features of the tumour microenvironment such as oxygen and drug diffusion gradients, and cell-extracellular matrix (ECM) signalling. 3D multicellular tumour spheroids more closely represent tissue organisation *in vivo* and offer a powerful alternative to classical 2D models.

To elucidate the mechanisms of cell invasion and evaluate the effects of drugs on cell migration, spheroids need to be imaged in their entirety, live, over several hours. This brings new challenges in terms of sample preparation, microscopy, data analysis and interpretation. We here describe the use of a multicellular tumour spheroid model to investigate cellular migration and invasion in 3D using light-sheet fluorescence microscopy (LSFM). In a typical spheroid invasion assay, spheroids are plated on, or embedded in, ECM in a 96-well plate. The plates are imaged using an inverted microscope and invasion is quantified by measuring the distance that cells have migrated away from the spheroid (5, 6). While this technique has the advantage of being high-throughput, it only provides information about the movement of cells in the x-y plane and does not provide any information about the migration of cells within spheroids. A further disadvantage of this technique is the inability to track individual cells.

We here describe an experimental protocol to track the invasion of individual cells in the x, y and z planes using LSFM. Both the migration of cells within spheroids, as well as their invasion in to the surrounding matrix, can be studied. Such an approach provides important information about the effects of the 3D architecture of cells on cell migration. While it is possible to acquire 3D images using a confocal microscope, the depth penetration is poor. The development of LSFM, also known as single-plane illumination microscopy (SPIM), has led to a revolution in the imaging of large (> 0.2 mm) living biological samples in 3D. LSFM was developed by Huisken *et al.* in 2004 and uses a plane of light, provided by dual illumination objectives, to provide optical sectioning (7). The sheet of light illuminates only the focal plane of the detection objective, resulting in reduced photobleaching and phototoxicity compared to confocal microscopy (7, 8). LSFM proved very soon to be an ideal tool to rapidly observe large living multicellular specimens obtained in a three-dimensional cell culture (9)

Spheroids are embedded in a solution of matrigel and media and mounted in an FEP tube, which is suspended in a liquid-filled chamber where it can be moved through the light sheet to produce a z-stack. Light is detected by a CCD or CMOS camera, which allows for very fast image acquisition compared with traditional point-scanning confocal microscopy, which samples pixel by pixel. This makes LSFM an ideal technique for imaging cell movement in a large, tightly packed spheroid when z-stacks need to be acquired in rapid succession to facilitate the tracking of cells. To make analysis more amenable, the data can be down-sampled in space and bit-depth to allow further analysis on a range of computer hardware. From the raw or processed data it is possible to quantify the movements of cells within the spheroids, as well as those invading into ECM.

**2. Materials**

2.1 Cell Culture:

* U87 glioblastoma cells (HTB-14; ATCC, UK)
* T-75 flasks
* Minimum Essential Medium (MEM) supplemented with 10% v/v Foetal Bovine Serum and 1% v/v Sodium-Pyruvate
* We used U87 cells stably expressing Histone 2B fused to Red Fluorescent protein (H2B-mRFP) using lentiviral transduction. The plasmid used for transduction was pHIV-H2BmRFP (Addgene, plasmid #18982)

2.2. Materials

* 96-well Perfecta3D® Hanging Drop plates (3D Biomatrix, USA; ordered from Sigma) for spheroid formation
* 0.22 μm PES filters (Biofil®, Guangzhou, China)
* Teflon plunger (Brand GMBH, Wertheim, Germany)
* Glass capillaries with green tag, size 3, inner diameter 1 mm (Brand GMBH, Wertheim, Germany)
* Fluorinated Ethylene Propylene (FEP) tubing, 1/16"ID x 1/8"OD (S 1815-04, BOLA, Germany)
* Parafilm

2.3. Chemicals

* Corning® Matrigel® Basement Membrane Matrix, phenol red free (Corning, ordered from VWR, UK)

2.4. Equipment

* Light-sheet microscope Z.1 (Zeiss, Germany)
* Cell incubator set at 37 °C, 5% CO2 (Sanyo, Japan)
* TC20 automated cell counter (Bio-Rad, UK)
* HP Z640 computer workstation (Hewlett-Packard, US) equipped with six central processing units (CPUs; Intel Xeon E5-2620v3 2.4 GHz) and 128 GB of DDR4 RAM (See Note 1).

**3. Methods**

3.1. Cell staining for imaging and tracking

A nuclear marker is used to facilitate the automated identification of cells. We previously found that nuclear stains show poor penetration in spheroids; therefore, it was necessary to create a cell line stably expressing a fluorescent nuclear reporter protein. We expressed histone H2B by transducing U87 cells with the pHIV-H2BmRFP construct generated by Welm *et al.* (10) (see Note 2).

3.2. Formation of multicellular tumour spheroids

1. Make a cell suspension using a pellet of 2.4 x 104 U87 cells in 1 mL of filtered growth media. Media needs to be filtered through a 0.22 μm PES filter as unfiltered media contains small particulates that spheroids adhere to.

2. Pipet the cell suspension (50 μl per well) into a 96-well Perfecta3D Hanging Drop plate where it will form a hanging droplet (Figure 1). After three days, the cells will have compacted from a loose aggregate and formed a compact spheroid.

3. On day 3, transfer the spheroids to a 35 mm non-treated culture dish using a P1000 pipette. Depending on size/compactness of the spheroids, it is usually beneficial to cut the final segment of the blue P1000 tip to widen the diameter of the tip and avoid spheroid disruption during the transfer and change media every 2-3 days (2 mL media/dish). As spheroids settle at the bottom of the dish, the old media is simply aspirated with a pipette and replaced with fresh media.

*[Insert Figure 1 here]*

3.3. Sample mounting

1. Prepare all the material needed for spheroid mounting: FEP tubes, Teflon plunger and glass capillary (Figure 2A).

2. Collect individual spheroids from the tissue culture dish using a Pasteur pipette, and transfer in a droplet of media to an empty dish (Figure 2B).

3. Aspirate the surrounding media using a pipette and replace with 50% ice-cold Matrigel and 50% growth media supplemented with 25 mM HEPES. See Note 3 about the choice of Matrigel for embedding and Note 4 for drug treatment, if required.

4. Insert a Teflon plunger into an FEP tube (several cm in length) and use it to draw the spheroid into the opposite end of the tube (Figure 2C). Before drawing up the spheroid, a small amount of air needs to be drawn up to create an air gap where the tube can be cut using scissors to release it from the plunger.

5. Insert the FEP tube into a glass capillary which is marginally wider in diameter than the FEP tube (Figure 2D). If a close fit is achieved, there is no need for any additional fixative to hold the tube within the capillary.

6. Wrap the end of the FEP tube, which is not inside the glass capillary, in parafilm (Figure 2E), leaving the sample (red arrow) uncovered (Figure 2F), insert the complete set-up in the standard Zeiss sample holder (Figure 2G) and immerse in the sample chamber, which is filled with RO water and maintained at 37 °C for the duration of the experiment.

*[Insert Figure 2 here]*

3.4. Image acquisition

1. Excite the samples with the appropriate wavelength for the fluorophore used, using two10x illumination objectives. Collect emitted light through the appropriate filter for the fluorophore using a 20x W Plan-Apochromat detection objective.

2. Acquire images every 3 min with a z spacing of 1.66 μm, starting approximately 50 μm in front of the spheroid. Use the Lightsheet Z.1 Zen software (Zeiss) for image acquisition (Figure 3). Acquisition can be performed from three angles if necessary for further 3D reconstruction, depending upon sample size (see Note 6). Images are acquired using a pco.edge scientific complementary metal–oxide–semiconductor (sCMOS) camera.

*[Insert Figure 3 here]*

* 1. Image processing

We suggest using the following workflow to prepare the data for analysis (Figure 4). Pre-processing the data is essential if you do not have access to a computer cluster, due to the large file sizes produced (up to 0.75 TB for one experiment). Down-sampling is acceptable in our case, as the loss in resolution has minimal effect upon the precision of feature detection.

*[Insert figure 4 here]*

1. Use the macro written in Fiji by D. Mason (available at https://bitbucket.org/davemason/lsfm\_scripts). The macro loads one time point at a time and down-samples the data by decreasing bit depth from 16 bit to 8 bit and performing 2x spatial binning in X and Y. Options for cropping are also available. More down-sampling means a smaller file size and faster manipulation but less precise spot detection.

2. If you have used multiple acquisition angles (Note 6), you can register and fuse them using the Multiview Reconstruction Application plugin (11), following the tutorials on the ImageJ website (http://imagej.net/Multiview-Reconstruction also archived at: https://web.archive.org/web/20170222212102/http://imagej.net/Multiview-Reconstruction). If there is a problem with sample drift (see Note 7), the same plugin can also be used for drift correction.

* 1. Image analysis

After processing, the data are amenable to feature detection and tracking using the open source software Fiji (for example via the Trackmate plugin) or using other software such as Imaris. Simple statistics such as instantaneous spot speed are provided in the output, however bespoke analysis can be used to query the data in a multitude of ways.

**4. Notes**

**Note 1:** The computational requirements are offered as a guideline only and will be highly dependent upon your sample and acquisition settings. The down-sampling script referenced in 3.5.1 requires a single time point be loaded into memory at once therefore this should inform hardware requirements.

**Note 2**: The use of longer excitation/emission wavelength red fluorescent proteins (RFPs) results in reduced autofluorescence, light scattering and excitation phototoxicity, making RFP (and variants) the most suitable fluorescent protein variant for long-time-lapse live-cell imaging in thick samples (12).

**Note 3**: The most typical approach for preparing small samples for imaging using LSFM is to embed them in a hydrogel, such as agarose, which has a refraction index close to that of water. We initially attempted to embed spheroids in 1% (w/v) low-melt agarose diluted in cell culture medium. However, agarose is not an ideal embedding medium for long-term culture because it is not a physiological substrate; it also precluded cell invasion. We also tried embedding spheroids in collagen IV and MaxGel ECM (Sigma); however, we again found that the cells did not invade.

**Note 4:** If drug treatment is required, the drug can be added to the growth media at the desired concentration.

**Note 5:** FEP tubes: Spheroids were mounted in a Matrigel-medium mix in FEP tubes, thereby creating conditions suitable for stable long-term imaging. As the spheroid is contained in a discrete compartment the sample chamber can be filled with distilled water instead of media. This means that there is no need to dismantle and sterilise the chamber after each use as contamination is not an issue. Furthermore, much smaller volumes of expensive reagents (eg Matrigel, drugs) are required because the volume of the FEP tube is much smaller than the volume of the sample chamber.

**Note 6**: Multi-view acquisition and reconstruction: Many small samples (below ~150 μm) can be imaged from a single angle. Some small and many larger samples such as spheroids will benefit greatly from acquiring multiple acquisition angles and reconstructing them post acquisition.

While it is possible to carry out online dual side fusion using the Zen software, this does not include a registration step so can complicate multiview registration if the system is not perfectly aligned. We suggest that users who are doing more than one angle do not enable dual side fusion, and instead use the Multiview Registration Application to register both dual side data and multiple acquisition angles at the same time.

**Note 7**: Drift: We found that an initial sample drift, typically upward, tended to occur in the first three hours of the experiment, before cells had begun to invade. Therefore, we chose to mount spheroids on the lightsheet microscope immediately after embedding but to delay imaging for three hours after sample mounting, to allow the sample to stabilise.

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**Figure Legends:**

**Figure 1: Hanging Drop plate**. The cell suspension is pipetted into the wells to form a hanging droplet (black arrow). Spheroids forms within 3 days in the droplets. The spheroids are harvested by tapping the plate onto a large petri dish, so that they can be pipetted and transferred into a 35 mm dish filled with cell culture medium.

**Figure 2. Spheroid mounting**. (**A**) From the top: ruler shown for scale, glass capillary (left) and FEP tube (right), Teflon plunger (**B**) single spheroid (red arrow) in a droplet containing the matrigel-medium mix. (**C**) Drawing of the spheroid (red arrow) into the FEP tube. (**D**) Detail of FEP tube held within the glass capillary. (**E**) Sealing one end of the FEP tube with parafilm. (**F**) Detail view of the spheroid (red arrow) in the FEP tube. (**G**) Photograph of the whole capillary holder ready to be inserted into the LSFM microscope.

**Figure 3. Image acquisition.** Select the Z stack, Time Series and Multiview options in Experiment Manager (**A**).Switch on the laser lines needed (**B**). Set up the appropriate lightpath (**C**) laser intensity and exposure times (**D**) for the fluorescent labels in use. Decide the duration of the experiment and the time interval between the acquisitions (**E**). Set the z spacing (**F**): 1.00 micron in the example. Select the desired number of rotations with the “Quick Setup” option (**G**), with three angles (In the example: 0, 120, 240 degrees as suggested minimum.

**Figure 4: Summary of steps to reduce file size and make data sets ready for image analysis**. After acquisition, Fiji is used to first reduce file size by down-sampling and binning, and then to correct sample drift and register the multiple views. Approximate file sizes after each step and time to process are shown for a large spheroid imaged from three angles, although these are highly sample dependent.