PROCEDURES TO CULTURE AND ANALYZE CELLS ON NANOFIBER SCAFFOLDS

Cultures prepared with these protocols have been established and studied as shown in Agudelo-Garcia et al., *Glioma cell migration on three-dimensional nanofiber scaffolds* [...]" Neoplasia (2011) 13 (9): 831-840

Protocol 1: Preparing nanofibers for cell culture

1. Remove nanofiber plate from the sterile pouch and place inside a biosafety hood
2. Rinse the plates 2x with sterile water. Allow the fibers to air-dry
3. For cells that have low attachment to plasticware (e.g: neural and tumor stem cells), coat the nanofibers with a suitable adhesive ECM protein, e.g. fibronectin (5-10 µg/ml) or laminin-1 (5-20 µg/ml), for 2h at room temperature (alternatively coating can be done overnight at 2-8C)
4. Rinse the plate 2x with sterile PBS (100 mM phosphate buffered saline solution) and 1x with the desired culture medium. The scaffolds are now ready for cell culture. Cells can be dissociated by conventional methods and pipetted into the wells or cell spheroids can be applied manually

Recommendations:

Culture plate	Volume for coating solution	Initial cell density	Culture volume
96-well plate	40 µl / well	1,000 - 5,000 /well	100 µl
24-well plate	200 µl / well	5,000 - 20,000 /well	500 µl

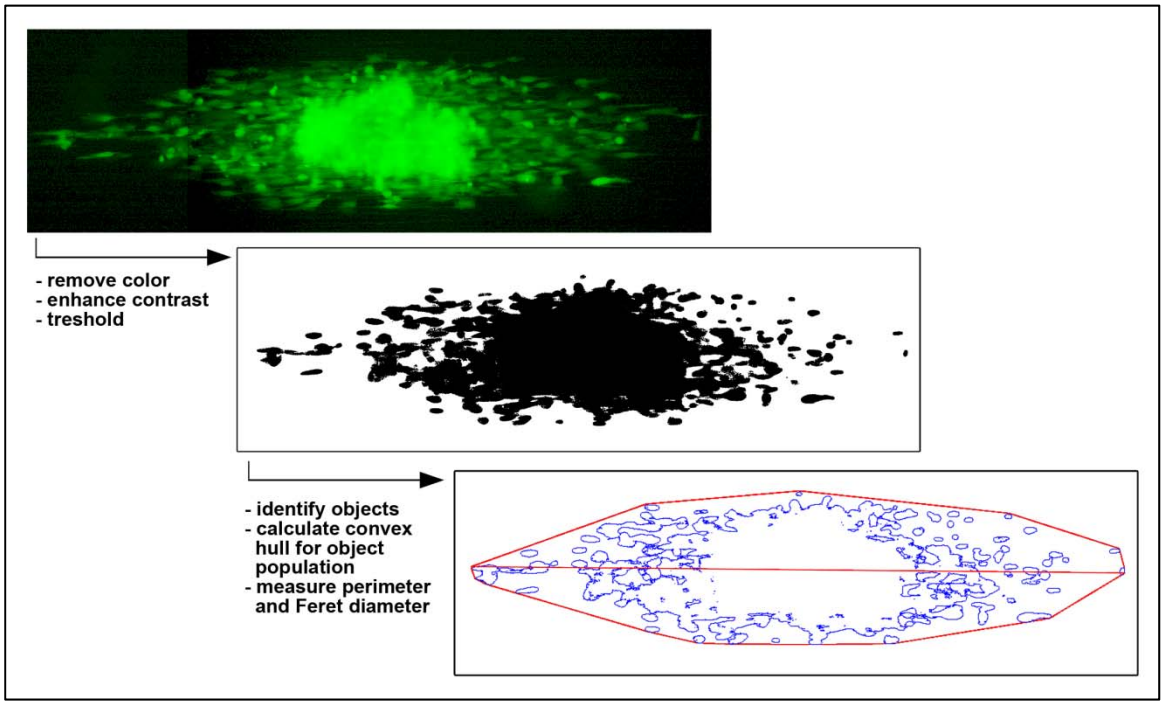
Protocol 2: Preparation and culture of cell spheroids on nanofibers

Cell spheroids are prepared by culturing dissociated cells on non-adhesive surfaces. Examples include culturing dissociated cells on hydrophobic cultureware (e.g., Corning Flat Bottom Ultra-Low Attachment Microplates, cat #3474) or culturing on 1% hard agar-coated plates (see Protocol 6 below).

1. Culture cells on hydrophobic surfaces until spheroids of 200-300 µm diameter can be easily observed under the microscope
2. Transfer the spheroids to a different vessel or conical tube and stain them with a tracking dye for 60min at 37C following the manufacturer's recommendations (recommended dyes: Cell-Tracker dyes, Invitrogen, used at 5 µM final concentration in culture medium)
3. Let the spheroids sediment to the bottom of the tube and remove as much of the staining solution as possible. Rinse the spheroids once with prewarmed culture medium
4. Transfer the spheroids to a 35mm or 60mm culture dish to manipulate them
5. Using a dissection microscope aspirate each spheroid individually (using a 200 µl tip) and seed them in individual wells of nanofiber-coated plates. Let the spheroids attach to the wells for 1h at room temperature in the biosafety hood (make sure the wells have sufficient liquid to avoid drying the spheroids)
6. Complete the culture medium in each well to the desired volume and return the plates to the culture incubator

Protocol 3: **Measuring axial cell dispersion on aligned nanofibers**

1. Seed individual, fluorescently-labeled spheroids on aligned-nanofiber plates and let them attach as indicated in Protocol 2
2. Using a fluorescent microscope take pictures corresponding to t=0h. Spheroids are easily detected by brightfield microscopy at this time, therefore they can also be imaged by phase brightfield microscopy
3. At the desired intervals, image the spheroids using fluorescence microscopy. It is essential that the spheroids are fluorescently labeled because dispersed cells on nanofibers cannot be reliably imaged using brightfield microscopy



(Figure reproduced from Agudelo-Garcia et al., Neoplasia (2011)13: 831-840, Suppl. Fig S1)

4. To calculate a *Cell Dispersion Index* at t=x measure the ratio $Fmax_{tx}/Fmax_{t0}$, where Fmax= Feret diameter of the cell population as shown in the Figure above (do not use total area because it is not a reliable measure depending solely on cell dispersion)

Note 1: Cell migration experiments should be performed for 24-48h. Longer migration times can introduce artifacts due to cell proliferation and loss of fluorescent dye.
Note 2: If addition of Cell-Tracker to the initial spheroids is a concern then the following protocol can be performed:

- Seed unstained aggregates and image them at t=0 using brightfield microscopy
- At the desired times post-migration add 1 μM Calcein-AM (Invitrogen) to each well and incubate it for 20 min at 37C.
- Rinse the wells once with culture medium and image the dispersed cells using fluorescence microscopy (Calcein-stained cells will be bright green)

Note 3: Do NOT add Cell-Tracker dyes or nuclear dyes (DAPI, Hoechst) to unstained aggregates once they are on nanofibers. The dyes will bind the nanofibers resulting in very high background for imaging.

Protocol 4: Immunocytochemistry of cells on nanofibers

1. Rinse the cells or spheroids on nanofibers using prewarmed PBS (with 1mM $CaCl_2$ and 1 mM $MgCl_2$)
2. Fix the cells with freshly prepared 100 mM phosphate buffered saline (pH 7.4) containing 4% paraformaldehyde and 0.1% glutaraldehyde to enhance cell fixation. Fix cultures for 10 min at room temperature. Avoid using organic solvents that can warp (methanol) or dissolve (acetone) the nanofibers
3. Rinse the cultures 3 x 5 minutes with PBS
4. Permeabilize the cells and block non-specific binding for 1h at room temperature using 100 mM PBS containing 0.3% v/v Triton X-100 and 5% w/v serum bovine albumin or other suitable blocking agent (consider increasing the concentration of blocking agent to prevent antibody adsorption to the nanofibers)
5. Perform ICC in the same conditions as for cells cultured on conventional cultureware
6. For low-magnification microscopy (2X-10X) cells can be stained and imaged in their original nanofiber-coated plates. For high-magnification microscopy (>10X) the backing material from individual wells must be cut ("punched out" using a metal puncher or cork borer) and the cut-out nanofiber disks can be mounted on glass slides or large rectangular coverslips.

Protocol 5: Cell recovery from nanofibers

1. To passage cells from nanofiber scaffolds to other cultureware the cells can be treated with trypsin, accutase, or other appropriate enzyme as if they were on conventional cultureware. Avoid using cell scrapers. Instead, gently dislodge the cells by careful pipetting and tapping on the culture plates
2. To prepare cell lysates the cells can be treated with the appropriate lysis buffer directly on the wells. Aqueous buffers without chaotropic agents (urea, guanidine HCl) will not affect the fibers.
3. Lyse the cells for 20 min on ice and scrape the nanofibers with a pipette tip to prepare a total lysate in the well. Collect the lysate with a pipette and transfer to a suitable tube
4. Centrifuge the tube for 10 min at 10,000*g* to remove insoluble fibers and cell debris
5. To extract RNA/DNA, cells can be collected in tubes as indicated above or the RNA extraction solution (eg, Trizol or similar) can be added directly to the wells and recovered by pipetting (proceed as if using conventional cultureware). This will melt and dissolve the fibers, therefore, the cell/fiber suspension must be centrifuged (10 min at 10,000*g*) to remove insoluble debris before continuing with RNA/DNA extraction

Protocol 6: Preparation of agar plates to make cell spheroids

1. Prepare a solution of 1% w/v molecular biology-grade agarose in DMEM. This will require dissolving the agarose carefully by heating in a microwave or heated bath

2. Let the agarose solution cool down to ~50C and filter through a large 0.22 um filter to sterilize the solution. You may need to fraction the agarose in small volumes and pass each volume through a separate filter to prevent clogging
3. Rapidly pipette the sterile agarose solution in 35 mm culture plates (1.5 ml / plate). Let them cool and gellify in a biosafety hood
4. Wrap in Saran Wrap (groups of 4-5 dishes together) and store at 4C for up to two weeks. Discard plates if the agarose shows cracks after storage
5. To prepare spheroids, warm the agarose plates to room temperature in a biosafety hood and add 1 ml of prewarmed culture medium per plate
6. Let the medium soak into the agarose and discard the medium after 1h. Refill the plates with 1 ml fresh medium
7. Seed dissociated cells at 50,000 - 75,000 cells/plate and return to culture incubator. Monitor development of cell aggregates over time (24 - 72 h). Carefully dislodge cells with a Pasteur pipette if they form large chains, to enhance the formation of individual aggregates

----------000----------