Spheroid invasion assay “on a chip”.

Introduction

Our spheroid invasion assay on a chip is inspired by study: Ravid-Hermesh, O. *et al.* Analysis of Cancer Cell Invasion and Anti-metastatic Drug Screening Using Hydrogel Micro-chamber Array (HMCA)-based Plates. *J. Vis. Exp.* 1–13 (2018). doi:10.3791/58359. In our case 3D micropatterning is made using 3D Petri dishes (microtissues; #12-81) which generates 81 spheroids of uniform sizes and also served as starting point for our spheroid invasion assay “on a chip”.

Materials

- 12 well plate
- UltraPure Agarose (1 % and 2 % v/v in PBS)
- Collagen (Fibronectin)
- 3D Petri dishes (microtissues; #12-81)

Procedure

Spheroid formation

1. Pipette 600-700 µl of 2% melted UltraPure Agarose (v/v in PBS; Sigma) to 3D petri dish and let solidify i to form micro-mold with 81 circular recesses (9 x 9 array; 800 µm each in diameter)

   You can speed up the process by 4°C (2-3 min), 3D petri dishes can be re-used to make more micro-molds. 2% UltraPure Agarose can be prepared in advanced (sterilized by boiling), solidified and once needed melted again. Keep 3D petri dishes sterile.

2. Transfer agarose mold to **12 well plate** and ideally fix molds in central position by adding extra agarose (1 ml) around.

3. Wash/equalibrate 2x with medium (10-30 min at 37°C)

   You can start preparing cells during equalibration

4. Prepare cells and add 40 500 cells in **190 µl** (=~210 000 cells/ml) to micro-mold, wait 10-15 minutes to settle cells at the bottom by gravity (check visually).

   The amount of cells is cell dependent (here optimized for A2780). If cells harvested from confluent 6well, 1/6 (+ 5/6 media) usually leads to ~210 000 cells/ml.
   More cells can form spheroid easier, but less space will be left for invasion. If micromold is used only for generation of spheroids, then more cells is very beneficial (146 000-500 000/190 µl).

5. Carefully add excess of media (1-2 ml) and let 48 h to self-assemble into 3D microtissues in the shape of a spheroid. To increase the compactness of the spheroid, it is benefitial to change 10 % serum to 1 % on the second day of spheroid formation (±Dox if you study the effect of gene expression).

   CRITICAL This is again cell dependent. Some cells need longer than 48h, but in general 1 % serum helps to make spheroid more compact. Check by eyes before progressing to next step

Invasion

6. Prepare melted 0.8-1.0 % UltraPure Agarose (v/v in PBS; Sigma) and transfer to 42 °C water bath. This will be used later at step 10. This step is not neccessary, but can help to prevent collagen gel shrinking.

7. Prepare premix (stable > 1 month) for collagen matrix or use different protocol as you are used to.

Table2

	A	B	C
1	Collagen premix		
2	DMEM/RPMI 10x	4.5	ml
3	H2O	17.831	ml
4	NaHCO3 (7,5%)	2.762	ml
5	NaOH 1M	0.131	ml
6	HEPES 1M	0.75	ml
7	Antibiotics/Cipro	0.026	ml
8	total volume	26	ml

Premix can be used up to 1 month. The amount of NaOH has to be at the beginning empirically determined based on Collagen in order to polymerize. Here it is optimized for High density type I collagen (Corning, #354249). The range for 26 ml is 0.02-0.424ul (if you do not know, add rather more as it is ussualy not problem)

8. Prepare collagen matrix as you need (keep on ice) using premix as set bellow. Here is general composition which should not inhibit any type of invasion (ameboid, mesenchymal): final Collagen 1.25 mg/ml with 25 µl/ml of fibronectin.

Table1

	A	B	C	D	E
1	PRODUCTION USING THE PREMIX (1.25 mg/ml)				
2		for 1 chip		calculator	
3	premix	128.3	ul	**2334.8**	**ul**
4	high collagen 10.9mg/ml	25.0	ul	**455.0**	**ul**
5	H2O	61.0	ul	**1110.1**	**ul**
6	FN (1mg/ml na 25 ug/ml)	5.5	ul	**100.1**	**ul**
7	**total volume**	**219.8**	**ul**	**4000.0**	**ul**
8		with excess			
9	enter the desired volume (ul)				

If you need to increase collagen amount, decrease amount of water accordingly. You can also add here labelled Fibronectin 1/10 with 9/10 non-labelled. Final mix: X mg/ml Collagen, 1× RPMI/DMEM, 15 mM HEPES (750 mM), 8.5 mM NaOH (1 M), 0.4 % $NaHCO_3$ (7.5 %, Sigma), 5 µg/ml folic acid (usually additive of 10x RPMI/DMEM)

9. Carefully aspirate the media (use aspirator and small 10 µl tips) and carefully add 190 µl of collagen/FN mix to each micromold.

   CRITICAL aspirate whole media aroud micromold and then from micromold by pointing with small 10 µl tips towards the corner of the micromold (check by eyes!).

10. Add carefully the collagen mix and let it polymerize in the incubator at 37°C in a humidified 5% (v/v) CO_2 atmosphere (15-30 min).

    CRITICAL This point is critical as you can washout the speroids from the bottom of wells and you also want to exchange any residual media with collagen (if cells are not in contact with Collagen, they will not migrate in the gell!!!). Best approach is to add collagen mix (drop by drop) to the center of the micromold from just above the micromold.
    CRITICAL The temperature will influence the pore sizes of collagen, so do it equally for all micromolds

11. After Collagen gel polymerization, overlay the chip with 81 spheroids embedded in collagen/FN gel by 0.8-1% UltraPure Agarose (v/v in PBS; kept at 42 °C; Sigma) to prevent gel movement and shrinking.

    UltraPure Agarose kept at 42 °C (agarose melting point) is critical as higher temperature can damage cells and collagen. Work quickly.

12. Finally, overlay it with final media (± drugs/inhibitors, count also for the volume of the agarose)

13. If needed, image your spheroids now = time 0 (at least check visually that there are no cells outside spheroid at this point = aritifical invasion...

    I prefer to image 6 spheroids simultanously using 4x objective (phase contrast)

14. Let cells to invade for 24-72 h at 37°C in a humidified 5% (v/v) CO_2 atmosphere (or more as you needed) and image again (4x objective, phase contrast, brightfield + fluorescence). Alternatively you can image them in real time with 10x, but you need long distance working objective.

    CRITICAL Be sure that your images are sharp and without shadows. I usually make nice images of two spheroids in the center of the image (4x, Ph1) which I then use for macro-analysis (brightfield). If you cannot get nice sharp images, consider fluorescence imaging (calcein staining, gfp...)

Analysis

15. In order to make analysis un-biased, fast and easy to do I use Fuji macro (Spheroid migration macro_Brightfield _2.0.ijm).

    CRITICAL This macro is only as good as the acquired spheroid images. I usually make nice images of two spheroids in the center of the image (4x, Ph1) giving the same contrast which I then use for macro-analysis (brightfield). Images obtained with different contrast will inevitably leed to differnt numbers!!!!. Spheroids at the edges should be avoided as their contrast is changed by the light comming through the sides of 12-well plate!!! If you cannot get nice sharp images without shadows, consider fluorescence imaging (calcein staining, gfp...)

Spheroid migration macro_Brightfield _2.0.ijm

```
imgName=getTitle();
baseNameEnd=indexOf(imgName, ".tif");
run("Properties...", "unit=um pixel_width=0.432 pixel_height=0.432 voxel_depth=0.432");
selectWindow(imgName);
    run("Apply LUT");
    run("16-bit");
    run("Enhance Contrast", "saturated=0.35");
    run("Find Edges");
    run("Invert LUT");
    setAutoThreshold("Default no-reset");
    run("Convert to Mask");
    run("Make Binary");
    run("Despeckle");
    run("Set Measurements...", "area area_fraction limit redirect=None decimal=5");
    //setTool("polygon");
    //values are in um;
    makePolygon(314,274,316,398,314,494,306,562,282,640,274,650,320,680,566,692,680,692,764,692,798,688,772,644,758,592,766,348,770,320,818,242,694,240,562,256,374,254,314,228);
    waitForUser("set the area and press OK");// move over upper midle spheroid
    run("Measure");
    waitForUser("set the area and press OK");// move over lower middel spheroid
    run("Measure");
    close();
```

Link to macro

This macro is usually able to satisfactorily recognize and subtract the main spheroid mass (=non-invading cells, time 0) and provide total area (μm^2) of invading cells.

CRITICAL The size of your selected invasion area for analysis, depends how invasive your cells are - if not much, measure invasion area in the micromold microchamber. The microchamber ring can add extra artificial pixels and these pixels may need to be subtracted if you compare invasive/non-invasive cells (highly invasive cells will make micromold ring unvisible under brightfield).

16. Load image and press run. Collect the numbers in excell file

    Sometimes macro provides the total image area (μm^2),and the percentage of migrating cells, but from this you can easily to re-calculate the total area (μm^2) of invading cell. For different objectives you need to change pixel/µm conversion values.