Three Dimensional Spheroid Co-culture Invasion Assay

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[Abstract] The assay was developed to investigate the impact of stromal cells of different types (in our case breast cancer associated fibroblasts stably manipulated to modify expression of genes of interest) on the invasive capacity of epithelial cancer cells (in our case breast cancer cell lines) (Verghese *et al.*, 2013). Typical two dimensional invasion assays do necessarily account for the presence of extracellular matrix that is present around the stromal and tumour cells in vivo and therefore cellular behaviour within these cultures may be non-physiological. This spheroid assay was developed to attempt to replicate more closely the environment that is present around breast cancer stromal and tumour cells in actual tumours (Verghese *et al.*, 2013). Extra cellular matrix composed of both collagen IV and collagen I is included and fibroblasts and epithelial cells are given the opportunity to develop "physiological" interactions (Verghese *et al.*, 2013; Hooper *et al.*, 2006). The method was developed from Nowicki *et al.* (2008), and we have published data using it in Verghese *et al.* (2013).

Materials and Reagents

1. Epithelial cells and fibroblasts [see Verghese *et al.* (2013) for the type we have used, although many others may be applicable]
2. Growth factor reduced matrigel (BD, catalogue number: 356231)
3. Collagen-1 (BD, catalog number: 354236)
4. Fetal calf serum (FCS) (Sigma-Aldrich, catalog number: F7524)
5. DMEM (Life Technologies, Invitrogen™, catalog number: 31966)
6. MEM powder (Sigma-Aldrich, catalog number: M0268)
7. 1 M N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (pH 7.5)
8. $NaHCO_3$
9. Rabbit anti-cytokeratin (Abcam, catalog number: Ab9377)
10. Zymed antibody diluent (Life Technologies, catalog number: 003218)
11. DPX (Sigma-Aldrich, catalog number: 317616) (a mounting agent)
12. Mayer's haematoxylin (any supplier)
13. Scott's tap water substitute (any supplier)
14. Eosin (any supplier)

15. Formalin (any supplier)
16. Ethanol (any supplier)
17. Xylene (any supplier)
18. Collagen-1/Matrigel mix (see Recipes)
19. Collagen medium (see Recipes)

Equipment

1. Tissue culture incubator (37 °C, humidified air/5% CO_2) (Panasonic Corporation, Sanyo)
2. 35 mm petri dish (ibidi GmbH, catalog number: 81158)
3. E1000 Eclipse microscope (Nikon Corporation)
4. Superfrost Plus slides and cover slips (Menzel Glaser)
5. Access retrieval unit (The Menarini Group) (a machine performing automated antigen retrieval giving enhanced reproducibility)
6. Automated stainer (IntelliPATH) (a machine performing automated immunohistochemistry protocols giving enhanced reproducibility)

Software

1. Photoshop (ADOBE)

Procedure

A. Spheroid formation

1. Equal numbers of epithelial cells (MCF-7 cells) and fibroblasts that are stably manipulated were suspended in DMEM medium/10% FCS at a final concentration of 1 x 10^6 /ml. 25 µl of this was placed in the centre of a 35 mm petri dish and inverted.
2. 5 ml of DMEM with 10% fetal calf serum was added to the lid of the petri dish to prevent drying.
3. This was incubated at 37 °C (humidified air/5% CO_2) for 24 h to allow formation of spheroids.
4. The next day 300 µl of a collagen-1/Matrigel mix (maintained on ice) was added to each well of a 24 well plate and incubated for 1 h for the gel to solidify.
5. Cells that had formed tight spheroids (defined as those that could easily be “picked” in their entirety using a pipette) were harvested using a 200 µl pipette and placed in the centre of the well containing the gel.

Note: Modifications to cells that reduce the strength of their cell-cell interactions may impact negatively on this process.

6. An additional 300 µl of collagen-1/Matrigel mix was added on top of the spheroid and incubated for 1 h at 37 °C.
7. Finally 300 µl of DMEM was added to the well. This was then incubated for 48 to 96 h (37 °C; humidified air/5% CO_2).

B. Quantifying invasion

1. Phase-contrast images were obtained at 24, 48 and 96 h using an E1000 Eclipse microscope at a predefined magnification.
2. The invasive area of cells outgrowing from the spheroids was quantified relative to the area of the central spheroidal itself. Quantification of the area was performed using Photoshop.
3. The identity of invasive cells (epithelial or fibroblast) was assessed using immunohistochemistry.
4. For representative images, illustrative examples of quantification, and assessment of variation between individual assays, please see Verghese *et al.* (2013).

C. Assessing expression/cell identity within spheroids

1. The collagen gels containing the spheroids were removed from the wells and fixed in 4% formalin overnight.
2. They were transferred to 70% ethanol and subsequently processed for paraffin embedding.
3. Multiple 5 µm sections of the collagen gel spheroids were prepared on to Superfrost Plus slides.
4. Every 5^{th} section from each spheroid was dewaxed in xylene and rehydrated with graded ethanol.
5. They were then stained in Mayer's haematoxylin, Scott's tap water substitute, and eosin.
6. Following this, sections were dehydrated in ethanol and xylene, and mounted in DPX. They were examined to identify the level at which the spheroids has the greatest diameter.
7. The sections before and after this level were used to perform immunohistochemistry to examine spheroid histology and expression within cell types of interest.
   a. We used rabbit anti-cytokeratin, a broad-spectrum anti-cytokeratin antibody, to label specifically the epithelial cells.
   b. The antibody was diluted at 1:100 in Zymed antibody diluent.

c. Antigen retrieval was performed using Access Revelation Solution in the Access retrieval unit and staining using an automated stainer (IntelliPATH running a standard X-Cell plus protocol). (Other antigen retrieval protocols are also suitable.)
d. Slides were counterstained in haematoxylin, mounted in DPX and images taken using the E1000 Eclipse microscope.
e. See Verghese *et al.* (2013) for illustrative examples.

Recipes

1. Collagen-1/Matrigel mix

   The following components were maintained on ice and mixed for a final Collagen-1 concentration of approximately 4.6 mg/ml and a final Matrigel concentration of approximately 2.2 mg. The final concentrations of collagen and matrigel were based on previous work (Gaggioli *et al.*, 2007). During the preparation of the mixture one should ensure all of the material (particularly collagen and matrigel) is pipetted successfully, rather than retained within the pipette tip. The final mix should be maintained on ice. We prepared this mixture freshly for every experiment and attempt to use only single batches of reagents since batch-to-batch variation can be an issue.

   200 µl Matrigel
   400 µl Collagen-1
   80 µl collagen medium
   100 µl fetal calf serum (FCS)
   220 µl DMEM
   Up to 1,000 µl with water
2. Collagen medium

   2.5 g MEM powder (5x)
   5 ml 1 M HEPES (pH 7.5)
   1 g $NaHCO_3$
   Up to 50 ml with water
   Mixed and then sterilised by passing it through a 0.2 µm filter

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References

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