Human Tumour Tissue Based 3D *in vitro* Invasion Assays

Running head: Human tissue based 3D *in vitro* invasion assays

Pirjo Åström, Ritva Heljasvaara, Pia Nyberg, Ahmed Al-Samadi and Tuula Salo

Abstract

Here we describe a protocol to utilize human benign leiomyoma tissue in *in vitro* 3D model that enables an assessment of cell invasion. The chapter also describes detailed instructions for image analysis to quantify the results. Leiomyoma is a benign tumour of the uterus which mimics authentic components of the tumour microenvironment including fibroblasts, vessels, collagen fibres, and extracellular protein composition. The leiomyoma invasion model represents a superior 3D model for cell invasion studies compared to the other non-human organotypic models.

Key words

3D *in vitro* model, invasion, human tissue, leiomyoma, extracellular matrix, tumour microenvironment, immunohistochemistry, image analysis, cancer cells, co-culture.

1. Introduction

Extracellular matrix (ECM) protein mixtures of non-human origin, such as Matrigel from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells are the prevailing substrates in the majority of *in vitro* cell invasion studies [1]. We have recently described a novel protein extract made of human uterus leiomyoma tissue and demonstrated that it is superior to Matrigel in *in vitro* cancer and angiogenesis studies [2]. However, such protein mixtures lack the complex 3D architecture which also influences proteolytic actions during cell invasion [3]. The described organotypic leiomyoma 3D solid disc model utilizes human tissue material with authentic components of the tumour microenvironment (TME) such as fibroblasts, insoluble and soluble ECM components, and a complex tumour tissue architecture including nerves and vessels [3, 4, 5]. Although cells in the myoma discs are not viable, their presence is important as they simulate the *in vivo* tumour cellular environment [6]. Additionally, myoma discs are hypoxic providing natural surroundings for cancer cells *in vitro* [3]. Hence, in this human 3D organotypic model, tumour progression and cell invasion can be investigated *in vitro* in an environment that closely mimics the *in vivo* situation.

2. Materials

Human uterine leiomyoma tissue can be obtained from routine surgical operations after the informed consent of the donors and kept frozen. Their use should be approved by ethical guidelines and regulations that follow international, national and institutional standards in each country, with the strictest legal and ethical standards.

Like all human tissues, the human leiomyoma specimens are potentially biohazardous material and thus they should only be handled by trained personnel. Processing of the myoma samples must be performed in dedicated areas, in a laminar hood with biosafety level 2. A full risk

assessment should be performed on all steps of the protocol. Human samples should be stored in designated areas, and their disposal should comply with institutional guidelines.

The procedure needs the following materials:

1. Human uterine leiomyoma tissue
2. Cultured tumour and other stromal cells
3. Cell culture reagents (depending on the cell type)
4. Transwell chambers (sterile 6.5 mm Transwell polycarbonate membrane inserts with an 8.0 μm pore size)
5. Slicing device or a sterile knife
6. 8 mm biopsy punch
7. 24-well and 12-well plates
8. Sterile tweezers (blunt and sharp)
9. Cryo tubes
10. Custom-made sterile metal grids
11. Microscope equipped with camera
12. Standard histology reagents for embedding and staining

3. Methods

The myoma invasion assay takes 10-14 days:

Day 0: Transfer myoma pieces to Transwell inserts, seed cells on top of the myoma

Day 1: Transfer myomas onto the metal grids

Day 3 - day 13: Change the media according to the culture protocol of your cells

Day 10-14: Collect the myomas for analyses and continue to further sample processing and analyses

3.1. Preparing the myoma discs for organotypic culture

1. Cut the leiomyoma tissue into 4 mm thick slices using a slicing device or a large knife in a sterile fashion (Figure 1A, B, see **Note 1**).
2. In a cell culture laminar hood, make cylinder-shape discs from the 4 mm leiomyoma slices using an 8 mm biopsy punch (Fig. 1C).
3. Store the discs at -70°C or in liquid nitrogen in cryo tubes filled with 1000 µl of the desired culture medium with 10% DMSO (see **Note 2**). Alternatively, the myoma discs can also be lyophilized (see **Note 3**).

3.2 Invasion assay (Day 0)

1. Remove myoma discs from the freezer or liquid nitrogen, thaw them and immerse the discs into the desired culture medium in a 50 ml Falcon tube (8 discs in 30-40 ml of medium). Rinse overnight at room temperature (or if the tissue is bloody, for up to 3 days at +4°C). Prepare at least three parallel myoma discs per experimental condition.
2. Use sterile blunt tweezers to transfer myoma discs gently into Transwell inserts placed in a 24-well plate avoiding tissue damage (Fig. 1D, see **Note 4**).
3. Immediately after the transfer, add 0.5 ml of culture medium to the lower chamber. Put the plate into cell culture incubator while preparing the cells.
4. Trypsinize the cells according to the protocol used for your cells.
5. Resuspend the cells and count the cell number. Suspend the cells in an appropriate volume of cell culture medium (*e.g.* for HSC-3 oral squamous cell carcinoma cells approximately 1.4×10^7 cells per ml is a good cell density). For co-culture experiments see **Note 5**.
6. Add tumour cells on top of the myoma in 50 µl of their normal culture medium (See **Note 6**).
- 6). The appropriate number of the cells depends on the cell line used, *e.g.* for HSC-3 cells,

700 000 cells per myoma disc is recommended. Also include myomas without cancer cell seeding in the study (control).

8. Culture at the appropriate conditions overnight.

3.3 Transfer the myomas onto the steel grids (day 1) and culturing (until day 10-14)

1. Place sterile metal grids in the wells of a 12-well plate.
2. Add 1 ml of culture medium (with or without test substance, *e.g.* cancer drugs) to the wells.
3. Take myoma discs from the Transwell inserts and place them on the grids (Fig. 1E).
Make sure that the cells are on the upper side of the myoma disc.
4. Culture at suitable conditions for up to two weeks, changing the media (1 ml) at desired intervals depending on the cell line (Fig. 1F). See **Note 7**.

3.4 Processing of the discs for analyses (day 10-14)

1. Prepare the tissues for histology.
 - a. Paraffin processing: Remove the myoma discs from the metal grids and place them in plastic cassettes of a suitable size (see **Note 8**). Immerse tissues in 4% neutral buffered formalin fixative for a maximum of 24 h, longer incubation can destroy antigens. Use gentle agitation, *e.g.* a small magnetic stirrer placed in a plastic cassette, that will enhance penetration of fixative to tissues. The volume of the fixative solution should be approximately 50-fold the volume of the tissue.
 - b. Frozen sample preparation: Slice the myomas into two halves and freeze the myoma tissue quickly in liquid nitrogen or place the disc in a plastic cryomold (in the correct orientation for cutting) and cover with optimal cutting

temperature compound (OCT) and rapidly freeze on an aluminium plate on the dry ice.

2. Place the cassettes under running tap water for 4 hours. Immerse the tissues in 40% EtOH for at least 4 h, and subsequently in 50% EtOH overnight, in 70% EtOH for 1 h, in 95% ethanol for 2 h, and finally in 100% EtOH for 2 h, all steps should be done under gentle agitation.
3. To remove all EtOH, immerse the tissue in xylene for 30 min under agitation.
4. Cut the myoma disc in two halves, orientate each halve in the cassette, embed it in paraffin and place the cassette on a cold plate to solidify.
5. Cut 5–6 μm myoma sections and stain, for example, with Hematoxylin and Eosin for tissue morphology (Fig. 2A). Immunohistochemical staining, *e.g.* anti-pancytokeratin antibody AE1/AE3 can be used to stain the carcinoma cells (Fig. 2B).

3.5 Analysing the invasion

The invasion area and invasion depth can be examined using image analysis software. Here we describe the use of Fiji software for analysing the invasion area and depth. However, there are also several other ways to apply Fiji software in image analyses [7].

1. Acquire 3-5 images of the whole invasion area (5x objective) or scan the area with a whole slide imaging scanner. Avoid the areas right next to the myoma edges during the image analysis (see **Note 9**).
2. Open the image and set the scale. Start by opening the Imagej.jar- file. Open the image from *File* $\rightarrow$ *Open* menu, or take the picture from a folder and drag it on the ImageJ symbol which will open the image. You can set the size of the image once you have opened it: Choose a tool for drawing a straight line from the menu bar and draw a line of known length. Select *Analyze*

→ *Set Scale*, insert the known distance value and its unit and select *Global*, so that the same scale will be used also in the following image that are opened (Fig. 3A).

3. Prepare the image for measurements. Select *Image* → *Type* → *8-bit*, to change the picture into an 8-bit grey shaded image. Select *Image* → *Adjust* → *Threshold* and use the mouse to adjust the scroll bars so that the regions to be studied are coloured red (Fig. 2B).

4. Define the epithelium and invasive regions. Select the Wand (tracing) tool with which you can define a uniform area. Click on the epithelium to draw/create a yellow line around the selected region. Select *Analyze* → *Analyze Particles*. You can select the minimum and maximum size of the particles to be measured on the part *Size* (μm^2). Select “*Ok*” to measure the surface area of the selected regions (Fig. 3C). Select *Edit* → *Clear*, to remove the already measured region from the image (Fig. 3D). Continue by drawing a line around the invasive region, or you can also select *Edit* → *Selection* → *Select all*, if the scale bar has been removed from the image. Select *Analyze* → *Analyze Particles* → *Ok*, to measure the surface area of the selected regions/particles. Now the surface areas and number of particles within the area are shown in the *Summary* window (Fig. 3E).

5. Measuring the depth of invasion. Adjust the settings for the measurements *Analyze* → *Set Measurement*, from here you only select *Limit Threshold* and *Display Label*. Select the ‘*Straight*’ tool from the menu and draw a vertical line starting from the lower threshold of the epithelium and ending at the particle that invaded the deepest/furthest (Fig. 3F). Select *Analyze* → *Measure* to obtain the result of the measurement in the *Results* window. Measure the invasion depths for three separate invasive particles with the deepest/furthest invasion.

6. Handling the results: The results can be copied from the *Summary* and *Results* windows straight into an excel-file. The average of invasion depth is calculated based on the measurements taken from three separate invasion particles.

4. Notes

1. In each individual experiment, use tissue discs from the same leiomyoma specimen. Special care should be taken when working with human tissues. All steps should be carried out in cell culture.
2. You can put up to 8 myoma discs in each cryo tube.
3. To lyophilize the myoma discs, first put them onto 24-or 48-well plates, one disc per well, cover with parafilm and freeze at -70°C . Pierce the parafilm and lyophilize the discs for 72 h using a Heto Drywinner DW3. After lyophilization, place the discs in an exicator. The discs are rehydrated by rinsing them at 4°C in the desired culture medium overnight. After rehydration, the discs can be used in the same manner as non-lyophilized native discs.
4. Myoma disc and the membrane should be packed tightly without any empty space between them to allow capillary flow of media into the myoma. If the disc is too high, cut it carefully to fit into the insert.
5. The Myoma *in vitro* 3D invasion model can be also used to co-culture cancer cells with other adherent and non-adherent cells within the tumour microenvironment.

A: Adherent cells. Cancer cells can be co-cultured with fibroblasts, macrophages, stem cells and other adherent cells by mixing the cells before adding them onto the myoma discs. The ratio between different cell types can be decided depending on the aim of the experiment and type of the cells. If needed, cells can be transfected with green fluorescent protein (GFP) so they can be differentiated from non-vital cells which exist within the Myoma disc.

B: Non-adherent cells. Cancer cells can be also co-cultured with non-adherent cells such as peripheral blood mononuclear cells by adding these cells in the medium below the myoma disc. These cells can also be activated by adding different stimulants such

as CD3 and CD28 antibodies, IL-2, ionomycin, phorbol 12-myristate 13-acetate, etc. to the medium. The number of the non-adherent cells can be decided depending on the aim of the study and the type of the cells.

6. In some cases, the medium flows on top of the myoma discs. Before adding the cells, remove any culture medium on top of the myoma.
7. When changing the media, conditioned media can be collected and stored at -20°C for various analyses *e.g.* for collagen degradation products during cancer cell invasion by radioimmunoassay kit.
8. Do not squeeze too big pieces of tissue into too small cassettes since this will result in poor penetration of fixative and possibly disturb the top cell layer.
9. Treat the images as research results. Save the original picture in TIFF form, JPEG is not recommended. Making simple adjustments is allowed (adjusting brightness, contrast and gamma – distribution). The settings must be identical when comparing images. Filters that improve image quality weaken the quality of the data. Intensities can be measured, if images have been modified as an entity and they have been calibrated for a known standard. You may lose some data while enlarging the image. Changes in the size of the image (number of pixels in X and Y axis) may cause folding artefacts.

References

1. Benton G, Arnaoutova I, George J, Kleinman HK, Koblinski J (2014) Matrigel: From discovery and ECM mimicry to assays and models for cancer research. *Adv Drug Deliv Rev* 79-80: 3-18.
2. Salo T, Sutinen M, Hoque Apu E, Sundquist E, Cervigne NK, de Oliveira CE, Akram SU, Ohlmeier S, Suomi F, Eklund L, Juusela P, Åström P, Bitu CC, Santala M, Savolainen K, Korvala J, Paes Leme AF, Coletta RD (2015) A novel human leiomyoma tissue derived matrix for cell culture studies. *BMC Cancer* 15:981.
3. Teppo S, Sundquist E, Vered M, Holappa H, Parkkisenniemi J, Rinaldi T, Lehenkari P, Grenman R, Dayan D, Risteli J, Salo T, Nyberg P (2013) The hypoxic tumor microenvironment regulates invasion of aggressive oral carcinoma cells. *Exp Cell Res* 319:376-389.
4. Nurmenniemi S, Sinikumpu T, Alahuhta I, Salo S, Sutinen M, Santala M, Risteli J, Nyberg P, Salo T (2009) A novel organotypic model mimics the tumor microenvironment. *Am J Pathol* 175:1281-1291.
5. Sundquist E, Renko O, Salo S, Magga J, Cervigne NK, Nyberg P, Risteli J, Sormunen R, Vuolteenaho O, Zandonadi F, Paes Leme AF, Coletta RD, Ruskoaho H, Salo T (2016) Neoplastic extracellular matrix environment promotes cancer invasion in vitro. *Exp Cell Res* 344:229-240.
6. Alahuhta I, Aikio M, Väyrynen O, Nurmenniemi S, Suojanen J, Teppo S, Pihlajaniemi T, Heljasvaara R, Salo T, Nyberg P (2015) Endostatin induces proliferation of oral carcinoma cells but its effect on invasion is modified by the tumor microenvironment. *Exp Cell Res* 336:130-140.
7. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K,

Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676-682.

Acknowledgements

This is a pre-print of an article published in Methods in Molecular Biology. The final authenticated version is available online at: https://doi.org/10.1007/978-1-4939-7595-2_19.

This work was supported by Sigrid Juselius Foundation, the Finnish Cancer Foundation, the University of Helsinki Central Hospital, and the Health Science Council of the Academy of Finland (Centre of Excellence 2012–2017 Grant 251314). We thank Dr. Sini Nurmenniemi and Dr. Tatu Pirkola for developing the myoma model and the protocol of invasion quantification respectively. We thank Mr. Donald Smart for the editorial assistance.

Affiliations

Pirjo Åström — Cancer and Translational Medicine Research Unit, University of Oulu, Oulu, Finland; Medical Research Center, Oulu University Hospital, Oulu, Finland; pirjo.astrom@oulu.fi

Ritva Heljasvaara — Oulu Center for Cell-Matrix Research, Biocenter Oulu, Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland; Centre for Cancer Biomarkers CCBIO, Department of Biomedicine, University of Bergen, Bergen, Norway; ritva.heljasvaara@oulu.fi

Pia Nyberg — Biobank Borealis of Northern Finland, Oulu University Hospital, Oulu, Finland; Medical Research Center, Oulu University Hospital and University of Oulu, Oulu, Finland; Institute of Cancer Research and Translational Medicine, University of Oulu, Oulu, Finland; pia.nyberg@oulu.fi

Ahmed Al-Samadi — Department of Oral and Maxillofacial Diseases, Clinicum, University of Helsinki, Helsinki, Finland; ahmed.al-samadi@helsinki.fi

Tuula Salo — Department of Oral and Maxillofacial Diseases, Clinicum, University of Helsinki, Helsinki, Finland; Cancer and Translational Medicine Research Unit, University of Oulu, Oulu, Finland; Medical Research Center, Oulu University Hospital, Oulu, Finland; tuula.salo@oulu.fi

Figures and legends

Figure 1. Selected steps for preparation of the myoma disc for invasion assay. (A) Cut the myoma tissue into 4 mm slices using a slicing device. (B) Myoma slices after cutting. (C) Cut myoma discs using 8 mm biopsy punch. (D) Insert the disc inside the Transwell chamber. (E) Transfer the disc from the Transwell chamber onto a metal grid. (F) Culture the myoma discs on the grids in a 12-well plate.

Figure 2. Histological sections of myoma discs with carcinoma cells after 10 days in culture. (A) Hematoxylin and Eosin staining. (B) Pancytokeratin AEI/AE3 immunohistochemical staining.

Figure 3. Selected steps for measuring the invasion area and invasion depth. (A) Open the desired image and set the scale. (B) Change the image to 8-bit depth and then adjust the threshold to get the stained area red in colour. (C) Use the tracing tool to select the area of non-invaded cancer cells. (D) Remove the non-invaded cancer cells and the scale bar from the image. (E) Summary of the results. (F) Measure the invasion depth by drawing a straight line from the bottom of the non-invaded cancer cells to the deepest point of the invaded cancer cells.