Chapter 20

3D Cancer Migration Assay with Schwann Cells

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Abstract

In pancreatic cancer, neural invasion is one of the most common paths of cancer dissemination. Classically, cancer cells actively invade nerves and cause local recurrence and pain. Three-dimensional (3D) neural migration assay has become a standard tool for scientists to study neural invasion by confronting the involved cell types. This protocol introduces Schwann cells, i.e., the most prevalent cell type in peripheral nerves, in a novel heterotypic, glia-cancer-neuron, 3D migration assay for assessing their relevance in the early pathogenesis of neural invasion. Particularly, this assay allows the monitoring of the early Schwann cell migratory activity.

Key words 3D migration assay, Schwann cells, Neural invasion, Pancreatic cancer, Dorsal root ganglia/DRG, Schwann cell carcinotropism

1 Introduction

The 3D neural migration assay is a special co-culture system aimed at studying neural invasion in pancreatic cancer [1]. Neural invasion can be defined as the continuous growth of tumor cells along nerves and is typically encountered around the epi- and perineurium of nerves. The cell types that are thought to play a major role in neural invasion (NI) are confronted simultaneously and photo-documented for 72 hours (h) in order to observe their migratory behavior, cell-to-cell interactions, and morphological changes.

In the original migration assay setup, these cell types were (a) pancreatic cancer (PCa) cell lines and (b) murine dorsal root ganglia (DRG), which were used to represent the neural component (Fig. 1). A characteristic feature of the present assay is that Schwann cells, as the most prevalent cell type in peripheral nerves, are added to the procedure as a third cell type, representing the glial component in the process of neural invasion. This addition revealed that, before cancer cells start to invade the DRG, Schwann cells migrate toward cancer cells due to their carcinotropic features [2].

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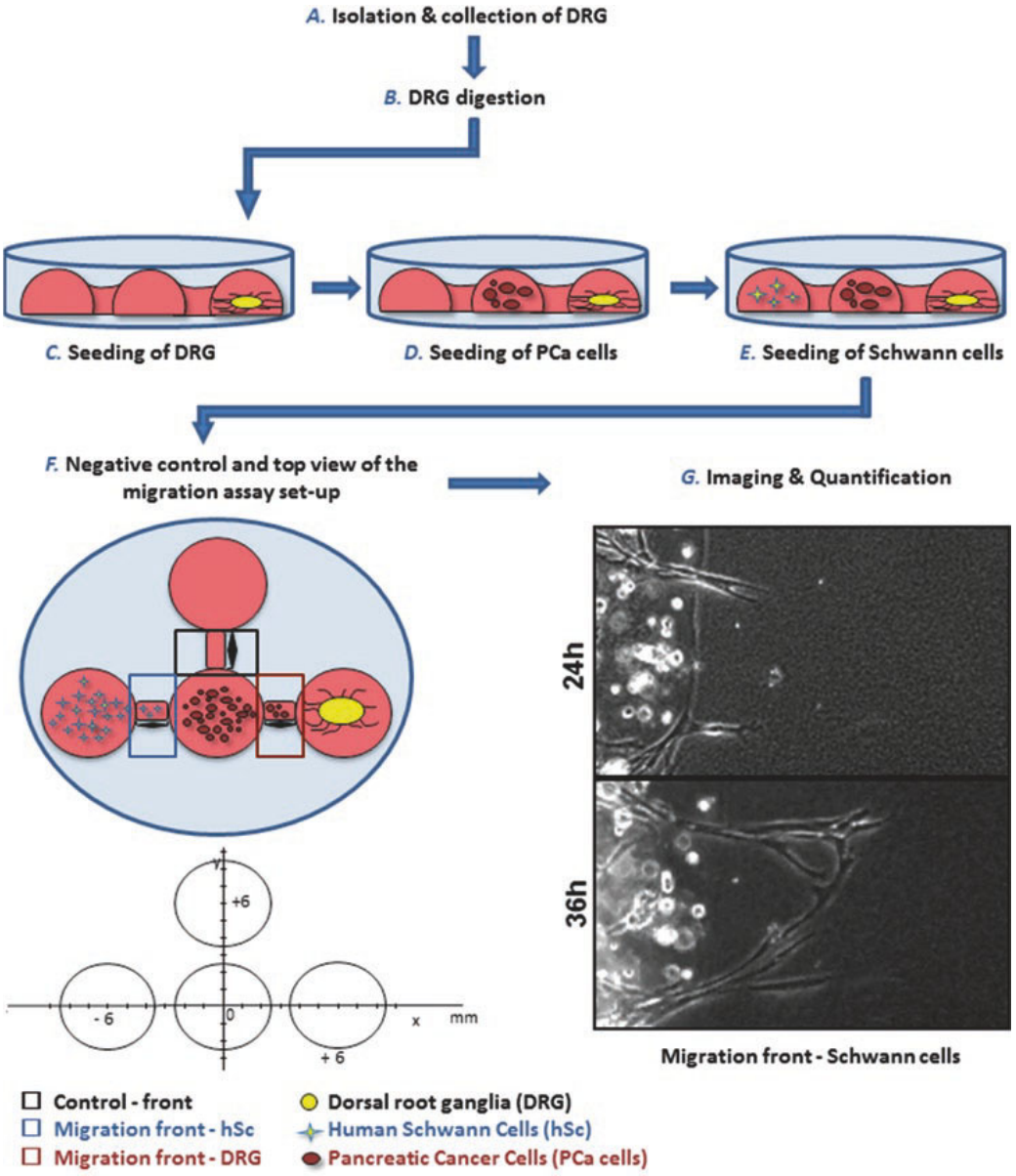

Fig. 1 (**a–e**) Overview of the pipetting steps for the 3D migration assay including pancreatic cancer cells (PCa cells), murine or rat DRGs, and human (or murine) Schwann cells (hSC). (**f**) Millimeter-scaled template designed as a pipetting scheme for the migration assay. First, place an ECM gel drop containing the DRG cells at millimeter +6 of a 35 mm culture dish. Next, place an additional ECM gel drop containing PCa cells at millimeter 0. Then, place the ECM cell drop containing the Schwann cell suspension at millimeter −6. Lastly, build an additional cell-free ECM gel drop at millimeter +6 of the *y*-axis serving as a negative control. (**g**) After 24 h, the migration of Schwann cells can be tracked via digital time-lapse microscopy and quantified for the extent of targeted migration

Therefore, the 3D migration assay with Schwann cells is suitable for simulating the mutual tropism between (a) PCa cells and DRG neurons, as well as (b) between glial cells, e.g., Schwann cells, and PCa cells [2]. Hence, in this triple-culture system, two sites of confrontation are present, i.e., first between PCa cells and DRG and

second between PCa cells and Schwann cells (Fig. 1). The cancer cell population additionally has a back front (Fig. 1) that is directed toward an empty gel suspension. All three cell populations are suspended in an extracellular matrix (ECM) gel, giving the co-culture system its 3D structure and placed in a culture dish at an exact distance of 1 mm by means of a mini-ruler beneath the culture dish. To allow migration, all three cell types are connected by an ECM gel bridge for establishing a chemoattractive gradient. After applying a defined neuronal medium, the assay is incubated for 48 h at 37 °C in a CO_2 incubator. At 24 h, the assay is taken out of the incubator and placed into a digital time-lapse microscope and photo-documented periodically for the time course of the experiment. The digital time-lapse microscopy allows exact tracking and quantification of the migratory activity. At 72 h after initial seeding of the assay, ECM gels containing each cell type are excised from the culture dish, dissociated, and lysed in radioimmunoprecipitation buffer for protein extraction. The extracted protein can be used for immunoblotting against, e.g., neurotrophic factor receptors to track their expression in migrating versus nonmigrating cancer or Schwann cells.

In summary, this assay shows that Schwann cells are attracted to cancer cells even before these start to migrate toward neurons. This observation suggests that Schwann cells serve as the first access for cancer cells during the process of NI, which ultimately causes tumor progression. Collectively, this chemoattraction of Schwann cells is termed Schwann cell carcinotropism [2]. Advantages of this system are the following: (1) the usage of a 3D cell culture model as opposed to a 2D culture; (2) the possibility to quantitatively assess cell migration by determining, e.g., the forward migration index (FMI), the migration velocity, and the linear (Euclidean) distance migrated by individual cells; and (3) the capacity to compare cancer cell migration toward different cell types within the same experimental setting.

2 Materials

2.1 Primary Cell Cultures and Established Cell Lines

1. Human PCa cell line SU86.86. This cell line can be purchased from American Type Culture Collection (ATCC) and should be cultured according to supplier's recommendations.
2. Human PCa cell line T3M4. This cell line is a kind gift by Dr. Metzgar (Durham, North Carolina) [1, 2].
3. Human Schwann cells. Primary human Schwann cells can be purchased from ScienCell Research Laboratories (Carlsbad, CA). Cells are derived from human spinal nerves. Cells can be cultured up to a maximum of ten passages when grown in complete human Schwann cell medium [1]. Alternatively,

murine (i.e., mouse or rat) Schwann cells provided by the same company can also be used in this assay.

4. Primary DRG neurons. Neurons are freshly isolated from newborn Wistar rats or C57BL/6J mice, as described below (*see* Subheading 3.1).

2.2 Preparation of Culture Media and Buffers

1. Human Schwann cell medium (ScienCell, Carlsbad, CA). The complete medium contains 5% fetal calf serum (FCS) and human Schwann cell growth supplements, as suggested by the manufacturer [1].
2. Neurobasal medium for DRG neurons. Neurobasal medium supplied with 100 U/ml penicillin, 100 µg/ml streptomycin, 2% B-27 supplement, and 0.5 mM L-glutamine.
3. Minimal essential medium (MEM, Sigma-Aldrich, Taufkirchen, Germany) supplied with 0.04 mg/ml gentamicin and 0.05 mg/ml metronidazole.
4. Hank's balanced salt solution (HBSS).
5. Gentamicin stock solution (10 mg/ml).
6. Metronidazole stock solution (5 mg/ml).
7. Collagenase type II. Dissolve 100 mg collagenase type II (Worthington Biochemical, Lakewood, NJ) in 10 ml HBSS to obtain a concentration of 10 mg/ml (10× stock solution). For digestion (*see* Subheading 3.1, **step 4**), add 100 µl of collagenase type II 10× stock solution and 900 µl of HBSS for a final collagenase concentration of 1 mg/ml (1× working solution). Store on ice.
8. ECM gel. It is recommended to use ECM from Engelbreth-Holm-Swarm (EHS) mouse sarcoma (E1270, Sigma-Aldrich, Munich, Germany). Thaw ECM gel on ice 1 h prior to experimentation. Keep ECM gel strictly on ice to avoid unwanted polymerization.
9. 1 M HEPES buffer solution prepared in water.
10. Radioimmunoprecipitation buffer (RIPA). 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl, 2 mM EDTA, 50 mM NaF. Adjust to pH 8.0. Add and dissolve one tablet of the protease inhibitor Complete (Roche, Penzberg, Germany) in the RIPA buffer for optimal protease inhibition.

2.3 Supplies, Equipment, and Software

1. 3.5 cm Petri dishes.
2. Millimeter-scaled template.
3. Micro-scissors.
4. Micro-forceps.
5. Hemocytometer.

6. CO_2 incubator (Heracell™). Set at a constant temperature of 37 °C and a humid atmosphere saturated with 5% CO_2.
7. Time-lapse microscope. Inverted light microscope (Zeiss Axio Observer D1) equipped with Zeiss CCD camera.
8. ImageJ software version 1.44 (NIH, Wayne Rasband) with manual tracking plug-in.
9. Chemotaxis and migration tool (Ibidi).

3 Methods

3.1 Isolation of DRG Neurons

1. Primary DRG neurons should be freshly isolated from three newborn Wistar rats or C57BL/6J mice between postnatal day (P) 2 and 12 after decapitation, anterior laminectomy, and stereomicroscopic dissection from the cervical to lumbar region (Fig. 2) [2] (*see* **Notes 1** and **2**).
2. Remove peripheral and central projections (nerve roots) of the DRGs by means of micro-scissors (Fig. 2) (*see* **Note 3**).
3. Collect DRGs in iced-cold MEM (Fig. 2).
4. Aspirate medium and resuspend the DRGs in HBSS supplied with collagenase type II and incubate them for 20–30 min, at 37 °C.

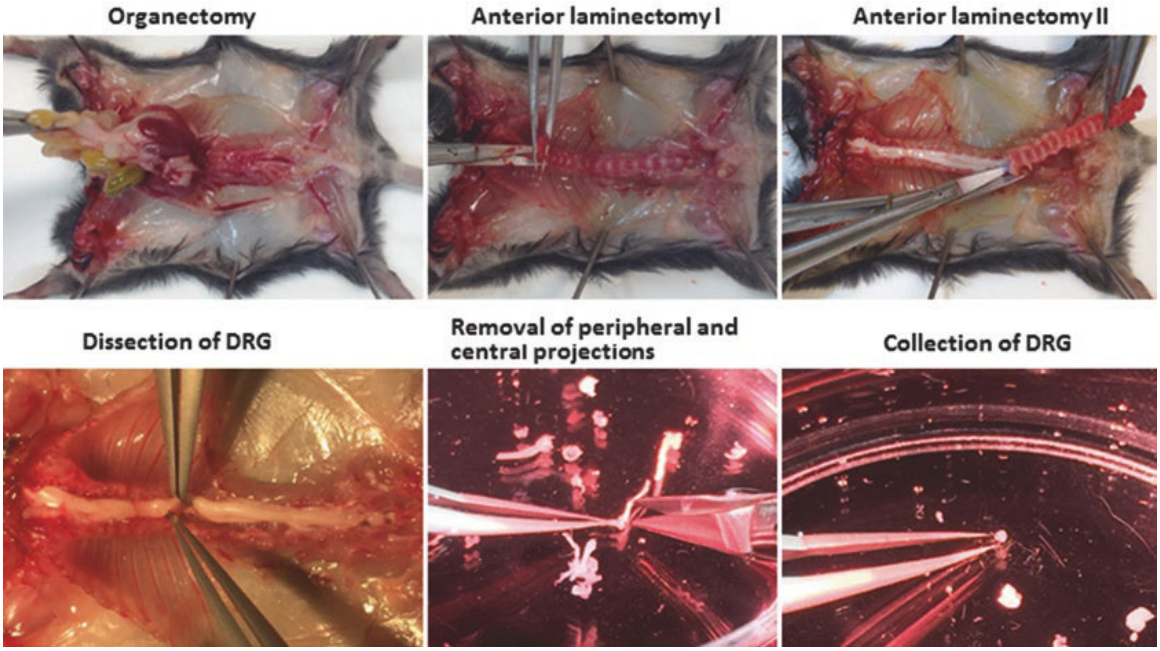

Fig. 2 Photographic depiction of DRG isolation from C57BL/6J mice. After removal of internal organs, an anterior laminectomy is performed for exposing the spinal cord. The collected DRGs are released from their projections to ease the subsequent trituration step

5. Triturate the DRGs through syringes of decreasing diameter (*see* **Notes 4** and **5**).
6. Centrifuge the DRG suspension at 93.9 × *g* for 5 min at 4 °C, aspirate old medium, and directly resuspend the cells in ECM gel. Use 25 μl of ECM gel per drop (*see* **Notes 6** and **7**).
7. Use a millimeter-scaled template for placing the ECM gel drop containing the DRG suspension in a 3.5 cm culture dish (*see* **Note 8**).
8. Incubate for 10 min at 37 °C, in a CO_2 incubator to allow polymerization of the ECM gel.

3.2 Preparation of Schwann Cells and PCa Cells

1. Count Schwann cells and PCa cells by means of a hemocytometer. The use of 10^5 cells of each cell type is recommended for one assay. Cells should be harvested for migration assay after reaching 100% confluence. The dissociation can be achieved by trypsinization in the culture flask.
2. Directly resuspend cells in ECM gel. Use 25 μl per drop (*see* **Notes 6** and **7**).
3. Use a millimeter-scaled template for placing the ECM gel drop containing PCa cell suspension in the culture dish, at an exact 1 mm distance to the left-hand side of the ECM gel drop containing the DRG suspension (Fig. 1) (*see* **Note 9**).
4. Repeat the procedure described above with Schwann cells. Place an ECM gel drop containing Schwann cell in the suspension at an exact 1 mm distance to the left-hand side of the ECM gel drop containing PCa cells (*see* **Note 10**).
5. In order to exclude unspecific cellular interaction, build an additional cell-free ECM gel drop and place it at an exact 1 mm distance to the upper front of the ECM drop containing PCa cells (*see* **Note 11**).
6. Incubate the dish for 10 min at 37 °C in a CO_2 incubator to allow polymerization of the ECM gel.

3.3 Setting Up the 3D Neural Migration Assay

1. Connect the ECM drops containing each cell type by building an ECM gel bridge in between adjacent ECM gel drops. Use 3 μl of ECM gel per bridge (*see* **Note 12**).
2. Incubate the dish for 15 min at 37 °C in a CO_2 incubator to allow polymerization of the ECM gel.
3. Cautiously add neurobasal medium for DRG neurons to submerge the ECM gel structures in a liquid phase of 3 ml of medium.
4. Incubate the dish for 48 h at 37 °C in a CO_2 incubator.

3.4 Time-Lapse Microscopy

1. Forty-eight hours after seeding the assay, add HEPES buffer (25 μl of HEPES buffer per ml of culture medium) for ionic stabilization purposes. Transfer the assay into the incubator of the time-lapse microscope and start the periodic photo-documentation every 15 min for up to 48 h.
2. Convert the obtained image sequences to an avi-formatted file compatible with the ImageJ software.
3. Using the manual tracking plug-in of the ImageJ, click on the cell to be tracked on every image of the image sequence. For this purpose, randomly select 30 cells at each migration front and track each cell with the manual tracking plug-in. The *x*/*y* calibration of the tracking plug-in should match that of the time-lapse microscope (e.g., 0.645 μm).
4. Save the file containing the results of the tracking as an .xls file and open it with the Ibidi's chemotaxis and migration tool for ImageJ.
5. Click on "Import data," enter the number of images in each tracked video, and select the statistic feature to obtain the summary of results. This analysis yields the following parameters: (1) the accumulated distance covered by migrating cells, (2) the Euclidean distance, (3) the velocity of migration, and (4) the FMI.

3.5 Protein Lysis for Immunoblotting

1. At 72 h after the initial seeding, excise the ECM gels containing each cell type from the culture dish and incubate them in HBSS containing collagenase type II (1 mg/ml) for 15 min to allow dissociation of the cells from the ECM gel.
2. Centrifuge the cells obtained after collagenase dissociation at 93.9 × *g* for 5 min. Aspirate the supernatant and add the RIPA buffer containing protease inhibitor for cell lysis.
3. Cell lysates may be subjected to immunoblot analysis together with samples of native Schwann cells or PCa cancer cells not subjected to co-culture. Analysis can be performed to determine the amount of neurotrophic factor receptors such as TrkA or p75NTR [2]. Analysis may show dynamic differences in the expression of such receptors upon co-culture of these cell types [2].

4 Notes

1. To achieve the best possible neuronal growth, it is recommended to avoid the use of mitotic inhibitors or additional neurotrophic factors in the DRG cultures. The above-described protocol confronts cancer cells with pure cultures of human Schwann cells on one side and with mixed neuronal-glial cul-

tures (murine or rat) on the other side. Mitosis inhibitors can also be applied to the ECM gel suspension containing the DRG cells to suppress glial cell division on the DRG side.

2. In order to have sufficient DRG neurons, collect all cervical to lumbar DRGs of each newborn rat or mouse (equaling 52 DRGs per rat/mouse).
3. Leaving the projections in place impedes trituration and increases the contamination risk of culture by fibroblasts.
4. For trituration, use 20 gauge/G needles followed by 23 G/ gauge needles. However, extensive trituration can negatively affect the viability of the DRG neurons more than the viability of glial cells. Typically, five to seven times of trituration with a 20 G needle and two times of trituration with a 23 G needle enable sufficient dissociation of the cells in the DRGs. Their viability can be confirmed with trypan blue staining.
5. ECM gel should be thawed on ice and kept on ice during the entire experiment to avoid early polymerization.
6. Strictly avoid air bubbles during resuspension of the cells in the ECM gel. Air bubbles decrease the quality of the migration assay.
7. It is recommended to use 35 mm culture dishes and place the ECM gel drop containing the DRG at millimeter 6 on the *x*-axis on the scaled template (Fig. 1f). The total diameter of one ECM gel plug should be 5 mm.
8. Carefully place the ECM gel drop containing the PCa cell suspension at millimeter 0 of the scaled template.
9. Carefully place the ECM gel drop containing the Schwann cell suspension at millimeter—6 of the scaled template.
10. Carefully place the cell-free ECM drop at millimeter 6 on the *y*-axis of the scaled template.
11. First, build a bridge for connecting DRG and PCa cells by placing 3 μl of ECM gel exactly in between these two drops. Build a second bridge for connecting PCa cells and Schwann cells in the same manner and finally a third bridge for connecting PCa cells with cell-free ECM drop.
12. The accumulated distance corresponds to the total path of cell migration. The Euclidean distance corresponds to the linear distance (net displacement) covered by migrating cells. The FMI is an index value equaling the proportion of Euclidean to accumulated distances and expresses the extent of targeted migration.

References

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