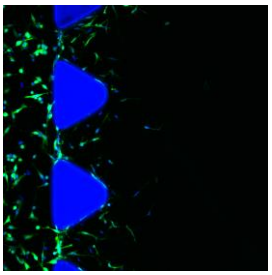
APPLICATION SPECIFIC PROTOCOL – CELL MIGRATION FOR ADHERENT CELLS

AIM idenTx 3 chips and idenTx 9 plates are very useful for the study of 3D cell invasion and migration. The idenTx 9 encompasses three individual idenTx 3 chips and a idenTx Holder, enabling 9 independent experiments on a single standard SBS-format plate. The chips and plate are not only suitable for endpoint measurement; they can monitor real time cell migration in response to a chemoattractant gradient, making cell trajectory information more readily available. The stimuli may include chemical entities, proteins, genetic regulators, mechanobiological factors, etc. The migration of breast cancer cells, MDA-MB-231 and fibrosarcoma cells, HT1080 are used here as illustrative examples.



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CELL INVASION & MIGRATION FROM THE GEL INTERFACE

Cells can be seeded on the gel interface and the invasion and migration of cells into the hydrogel can be triggered by the application of FBS or chemoattractant/s. In this setup, both real time monitoring and end point measurement of cell migration can be performed conveniently as the starting point of cell migration is at the medium-hydrogel interface.

SEEDING CELLS ⏱ TIMING 20 min

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 70011044)
- Trypsin (Life Technologies, Cat. No. 25300054)
- Cell culture medium (Life Technologies, Cat. No. 10569010)

Others

- Collagen-filled idenTx 3 or idenTx 9

1. Culture breast cancer cells, MDA-MB-231 in serum-free cell culture medium with 0.2% BSA or 0.2% FBS for 24 h before trypsinization.

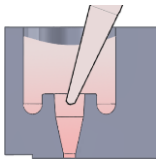
2. Trypsinize cells as per protocol and re-suspend the cells at 1.0 M cells/ml in serum-free cell culture medium.

3. Hydrate each of the media channels with 15 µL of serum-free cell culture medium and then top up each channel with 85 µL of serum-free medium (in total 100 µL of medium per channel).

Reminder *Ports must be filled with medium before seeding cells into the media channels.*

4. Add an additional 20 µl of serum-free medium into one of the ports at the media channel that is to be seeded with cells.

5. Use a micropipette to withdraw 10 µl of cancer cell suspension. Position the tip near the inlet of a media channel and inject the cell suspension. Wait for 2 min and then repeat the same procedure for the opposite connected inlet. In total, 20 µl of cancer cell suspension is seeded per media channel. The additional 40 µl of fluid (20 µl of cell suspension and 20 µl of medium) creates a height difference between the two media channels thus generating interstitial flow across the gel. This helps the attachment of cancer cells on the gel interface.



Position the pipette tip at media inlets while injecting cell suspension

6. Visual inspection under a microscope is recommended. If the cell distribution is not optimal for your application, adjust the concentration of the cell suspension and repeat the seeding steps.

! Critical *Do not insert the tip completely into the inlets to avoid introducing cells into the media channels at a high flow rate. High flows will not allow cells to settle along the channel, resulting in uneven distribution.*

7. Keep the chips in an incubator for 2 h before the application of chemoattractant/s.

! Critical *Lay chips (on idenTx holders or in humidified chambers) on a flat surface while seeding cells into idenTx 3 or idenTx 9. Inclination of the chips affects the cell distribution.*

? Troubleshooting (see Table 1 for troubleshooting advice)

CELL MIGRATION WITHIN THE HYDROGEL

Cells can be seeded within the 3D hydrogel and the migration of cells within the hydrogel can be triggered by the application of FBS or chemoattractant/s. This assay closely mimics *in vivo* conditions where interactions between cells and extracellular matrixes are accounted for. The 3D migration of cells can be tracked with real time imaging.

SEEDING CELLS ⏱ TIMING 20 min

MATERIALS

Reagents

- 10X PBS with phenol red as indicator
- Sodium hydroxide solution, 0.5 M
- Sterile deionized water (Thermo Water Purifying System)
- Collagen type I, rat tail (Corning Life Science, Cat. No. 354236)
- 1X PBS (Life Technologies, Cat. No. 70011044)
- Trypsin (Life Technologies, Cat. No. 25300054)
- Cell culture medium (Life Technologies, Cat. No. 10569010)

Others

- idenTx 3 or idenTx 9
- idenTx holders or humidified chambers

8. Culture fibrosarcoma cells, HT1080 in serum-free cell culture medium with 0.2% BSA or 0.2% FBS for 24 h before trypsinization.

9. Trypsinize cells as per protocol and re-suspend the cells at 10 M cells/ml in cell culture medium with 0.2% FBS.

10. Keep 10X PBS with phenol red, collagen stock solution, 0.5 M NaOH solution, deionized water and cell suspension on ice and add them into a microcentrifuge tube (on ice) sequentially according to the pre-determined collagen gel recipe (see the Protocol for Seeding Cells in Hydrogel).

11. Mix the solution thoroughly by using a micropipette to get a collagen solution with homogeneous faint pink color.

12. Draw 10 µL of collagen solution (with cells) with a 1- 10 µL micropipette. Make sure the collagen solution is kept on ice at all times.

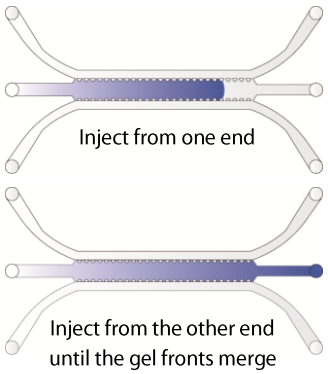
! **Critical** *Limit the volume of collagen solution to 10 µl to prevent the collagen solution from overflowing into media channels.*

13. Fill the chip from either gel inlet:

! **Critical** *Hold the plunger firmly while removing the micropipette from the inlets, otherwise the negative pressure will suck the collagen up.*

! **Critical** *Inject the collagen solution smoothly to ensure the cells distribute evenly in the gel.*

a. **Option 1**: Fill collagen solution from either one of the inlets and stop near the end of posts. Fill from the other inlet until the gel fronts merge. This method is recommended for new users.



b. **Option 2:** Fill collagen solution from one side all the way to the other side. Continue to push the collagen solution in gently until it reaches the other inlet. This method ensures that the gel is being filled homogeneously but it requires greater control over pipetting pressure (especially when the collagen solution reaches the opposite inlet) to prevent the collagen solution from overflowing into the flanking media channels.

14. Place the gel-filled chips (on idenTx holders or in humidified chambers) into a 37 °C incubator and incubate for half an hour to allow polymerization of collagen to take place.

Reminder *The polymerization time can be optimized to suit your specific application.*

! Critical *Temperature will affect collagen polymerization and 37 °C is recommended for most applications.*

! Critical *Chips with unpolymerized gel must be handled with care. Excessive agitation or impact may cause the unpolymerized gel to leak out of the gel channel.*

APPLICATION OF CHEMOATTRACTANT ⏱ TIMING 10 min

MATERIALS

Reagents

- Serum-free cell culture medium
- Cell culture medium with 10% (for cell invasion from the media channels) or 20% (for cell migration within the hydrogel) FBS or chemoattractant of choice (eg. EGF at 10 ng/ml)

15. Prepare the chips for the application of chemoattractant:
    a. Cell invasion from the media channels: Remove medium from all 4 ports by carefully aspirating medium out from the troughs.
    b. Cell migration within the 3D hydrogel: Hydrate each media channel with 15 µL of serum-free cell culture medium.

16. Add 70 µl of serum-free cell culture medium into one port of a channel and then add 50 µl to the opposite connected port. Repeat this for the other channel but use cell culture medium with 10 % FBS (for cell invasion from the media channels) or 20% FBS (for cell migration within the 3D hydrogel) or chemoattractant of choice to create a chemoattractant gradient across the collagen gel.
    a. Cell invasion from the media channels: Add the FBS-enriched medium in the opposite channel to the cell-populated channel.
    b. Cell migration within the 3D hydrogel: Add the FBS-enriched medium in one of the two media channels.

Reminder *The differential volumes in the two ports allow the replacement of medium to take place in the channel. The minimum volume of medium is 30 µl to ensure the inlets are covered and the troughs are wetted. If less than 30 µl of medium is used, the surface tension at the inlets will prevent the medium from flowing through the channel. We recommend using at least 50 µl of medium for easier handling.*

17. Keep the chips in an incubator. The invasion/migration of cancer cells into/in the 3D collagen should occur within 24 h after the application of chemoattractant.

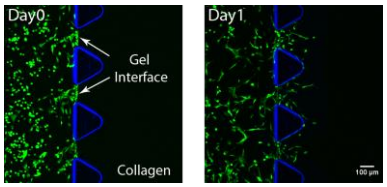


Figure 1 MDA-MB-231 breast cancer cells (green) pile up on the gel interface forming a clear starting point on day 0 and they invade into the 3D collagen after 24h.

Reminder *The gradient formed that is across the hydrogel will dissipate over time if it is not reset by a medium change or is not maintained by continuous flows in both channels.*

? Troubleshooting (see Table 1 for troubleshooting advice)

QUANTIFICATION OF CELL MIGRATION ⏱ TIMING Variable

In order to quantify the extent of cell migration in idenTx 3 or idenTx 9, we recommend labelling the cells with appropriate fluorophores to visualize them. Fluorescently-tagged cells (such as mCherry-tagged-HT1080) are especially useful for live cell imaging. Bright-field, phase contrast and epifluorescence microscopy are all compatible with idenTx 3 or idenTx 9 but three-dimensional imaging techniques such as confocal microscopy is preferred due to the nature of this assay. The following quantification methods use images taken from confocal microscopy as illustrative examples.

MIGRATION DISTANCE AND CELL NUMBER (IN END POINT ASSAY)

The migration distance of cells is an informative metric that can describe how fast (when the period of culture is taken into consideration) and how far the cells have travelled into the 3D gel region. For end point assay, we assume cell migration is unidirectional towards the chemoattractant as the travelling paths of cells are unknown. The number of invading cells also indicates the extent of the invasion.

18. We recommend using confocal images that capture the cell nuclei to quantify the migration distance and cell number.

19. Pre-process the images such as cropping the media channels out while retaining the 3D gel region is necessary only if the cells in the 3D gel region are of interest to your application. You should always pre-process your image as a whole.

20. Use Point Picker plugin (http://fiji.sc/Point_Picker) in ImageJ to first identify the edge of the gel interface (where the cells are seeded on Day 0) by choosing at least three random points along the gel interface. Calculate the average value of x-coordinates (y-coordinates if idenTx 3 or idenTx 9 are placed the other way round during imaging) from these three points.

! Critical *Make sure idenTx 3 or idenTx 9 is not slanted during image acquisition to minimize the errors in measuring the migration distance of cells from the gel interface.*

21. Use 3D Objects Counter plugin (http://fiji.sc/3D_Objects_Counter) in ImageJ to count the total number of nuclei and to obtain the individual Cartesian coordinates of every nucleus that has invaded and migrated into the 3D hydrogel region. Depending on the image quality, you may adjust the threshold level and size filter to make sure every nucleus in the region of interest is counted.

Reminder *We recommend validating this semi-automated counting method with manual counting (by using Point Picker plugin) as image quality can affect the accuracy of this counting method.*

22. Calculate the differences of the x-coordinates (y-coordinates if idenTx 3 or idenTx 9 are placed the other way round during imaging) between cells and the gel interface. Adjust the calculated numbers by using the scaling factor to obtain migration distances in the unit of length.

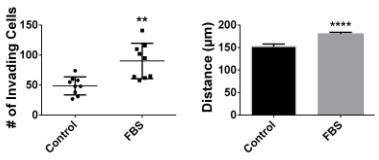


Figure 2 Scatter dot plot (left) of number of invading HT1080 cells into the collagen gel I and column bar graph (right) of the cell invasion distance. Higher number of cells invade into the 3D collagen gel and they migrate further towards the direction where 10% FBS is applied as compared to the control group (without FBS).

MIGRATION TRAJECTORIES OF LIVE CELLS

idenTx 3 or idenTx 9 is particularly useful in studying live cell migration within a 3D matrix where the migration trajectories of live cells can be monitored in real time. Based on the migration trajectories, the displacement and velocity of migrated cells can be estimated.

23. We recommend using confocal images of live cells that are either fluorescently tagged or stained in the nuclei to track their migration trajectories.

24. Pre-process the images such as reducing background noise through despeckle and background subtraction, if necessary. You should always pre-process your images as a whole.

Reminder *You may perform a 3D projection on a stack of images to generate a 2D image, only if the information across the different planes along the z-axis is not important for your application. The 3D projection helps reduce the computing power needed to process a time-lapse movie file with multiple planes.*

25. Use TrackMate plugin (http://fiji.sc/TrackMate) in ImageJ to track migration trajectories.

Reminder *You may use other particle tracking plugin that suits your application.*

Reminder *The tracking of cell migration trajectory is greatly affected by the choice of detector and particle-linking algorithms in the TrackMate plugin. Make sure the input values for detector (e.g. estimated blob diameter) and particle-linking algorithms (e.g. linking max distance) are representative of your data.*

26. You can derive the velocity and displacement for every captured cell based on the statistic of each trajectory. Alternatively, you can determine the directionality of cell migration by comparing the Cartesian coordinates between the last and first time points of the same cell to obtain the distribution of angles of net migration vectors.

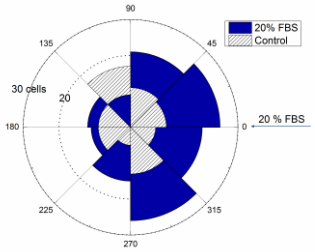


Figure 3 Polar histogram that demonstrates the distribution of angles of net migration vectors for cancer cells (HT1080) in the 3D hydrogel region. In the group with 20% FBS, the cancer cells migrate preferentially towards the direction where 20% FBS is applied. In contrast, no obvious directionality of cell migration is observed in the control group without additional FBS.

TROUBLESHOOTING

Table 1 Troubleshooting advice

Step	Problem	Possible Reason	Solution
6.	Cells do not distribute evenly	The interval between the injections of cell suspension is short thus the flow of cells in the channel may be disrupted	Wait for at least 2 min before seeding cells into the opposite connected inlet
6.	Too many cells in a channel	Concentration of cell suspension is too high	Flush out unattached cells with culture medium immediately and repeat the seeding steps with cell suspension that is less concentrated
6.	Too few cells in a channel	Concentration of cell suspension is too low	Increase the concentration of cell suspension or repeat the seeding steps (without modifying the concentration of cell suspension) until the target cell density is obtained
6.	Cells do not adhere to the gel interface	The pressure head applied is insufficient	Increase the volume of cell suspension
17.	No cell migration/invasion	Hydrogel is too stiff: Cells cannot invade into the gel	Optimize the concentration, gelling time and pH (only for collagen I) of the hydrogel
		Inappropriate amount of chemoattractant	Optimize the concentration of chemoattractant