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Cell Invasion in Direct Co-Culture

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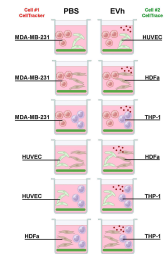
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We use this protocol and it's working

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Abstract

The fluorescent gelatin degradation assay is a method to study cell invasion by detecting gelatinase activity in vitro upon epifluorescence microscopy analysis. In this protocol, the method has been applied to evaluate the effect of hypoxic EVs from TNBC cell line MDA-MB-231 in a direct co-culture assay, using four cellular models for the tumor microenvironment. Hence, MDA-MB-231 (tumor cell), HUVEC (endothelial cell), HDFa (dermal fibroblast), and THP-1 (monocyte) have been seeded in six distinct combinations. Adapted from Pachane et al (2022) (PMID: 36293503).

Image Attribution

Experimental diagram created using BioRender.com and Adobe Photoshop.



Materials

Materials and reagents

1. Corning 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates, Individually Wrapped
2. Sterile microtubes and pipettes
3. Gelatin From Pig Skin, Fluorescein Conjugate, Thermo Fisher - Catalog #G13187
4. Sterile PBS
5. OptiMEM I Reduced Serum Media, Gibco - Catalog #31985070
6. Trypan Blue solution 0.4%, Merck Millipore (Sigma-Aldrich) - Catalog #T8154-100 ml
7. Paraformaldehyde solution (PFA 4% in deionized water, pH 7.6 - Sterile)
8. Triton X-100 0.1% (v/v) in deionized water
9. CellTracker™ Red CMTPX, Invitrogen - Catalog #C34552
10. CellTrace™ CFSE, Invitrogen - Catalog #C34554
11. Phalloidin + DAPI (1 µl Phalloidin-iFluor 647, Abcam - Catalog #ab176759 + 0.76 µL 4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI), Thermo Fisher Scientific - Catalog #D1306 in 5 mL PBS)

Cell lines and growth media

- MDA-MB-231 (ATCC® CRM-HTB-26™) - Leibovitz L-15 10% FBS
- HDFa (ATCC® PCS-201-012™) - DMEM 10% FBS 1% pen/strep
- HUVEC (ATCC® CRL-1730™) - DMEM 10% FBS 1% pen/strep
- THP-1 (ATCC® TIB-202™) - RPMI 1640 10% FBS 1% pen/strep

Equipments:

1. Biological cabinet
2. Cell incubator (37 °C, 5% CO₂)
3. Cell counter - TC20 Cell Counter, Bio-Rad - Catalog #1450011
4. Epifluorescence microscope - ImageXpress Micro XLS, Molecular Devices - Catalog #500496

Protocol materials



Parafilm

Safety warnings

- ! Light-sensitive assay. Work under sterile conditions.



Before start

Fluorescent gelatin preparation: Under sterile conditions, solubilize the fluorescent gelatin stock at $37\text{ }^{\circ}\text{C}$ with warmed PBS following the manufacturer's instructions for a concentration of 5 mg/mL . Aliquot in microtubes and maintain at $-20\text{ }^{\circ}\text{C}$ until time of use.

Before use, thaw gelatin at $37\text{ }^{\circ}\text{C}$ for $00:30:00$. Dilute stock to a 0.2 mg/mL working solution with warmed PBS and maintain at $37\text{ }^{\circ}\text{C}$ until use.

Cell culture: Maintain cells in culture during at least two passages after thawing.



Experimental Design and Plate Coating

- 1 Open a new 96-well black plate under sterile conditions and label groups in technical triplicates to contain a **vehicle (PBS) control** (i.e., untreated cells in OptiMEM) and the **EVh-treated group** (i.e., EVh-treated cells in OptiMEM) for each co-culture combination:
 1. MDA-MB-231 + HUVEC
 2. MDA-MB-231 + HDFa
 3. MDA-MB-231 + THP-1
 4. HUVEC + HDFa
 5. HUVEC + THP-1
 6. HDFa + THP-1

2

	1	2	3	4	5
A	MDA-HUVEC PBS	MDA-HUVEC PBS	MDA-HUVEC PBS	MDA-HUVEC EVh	MDA-HUVEC EVh
B	MDA-HDFa PBS	MDA-HDFa PBS	MDA-HDFa. PBS	MDA-HDFa EVh	MDA-HDFa EVh
C	MDA-THP1 P BS	MDA-THP1 P BS	MDA-THP1 P BS	MDA-THP1 E Vh	MDA-THP1 E Vh
D	HUVEC-HDFa PBS	HUVEC-HDFa PBS	HUVEC-HDFa PBS	HUVEC-HDFa EVh	HUVEC-HDFa EVh
E	HUVEC-THP1 PBS	HUVEC-THP1 PBS	HUVEC-THP1 PBS	HUVEC-THP1 EVh	HUVEC-THP1 EVh
F	HDFa-THP1 PBS	HDFa-THP1 PBS	HDFa-THP1 PBS	HDFa-THP1 EVh	HDFa-THP1 EVh
G					
H					
	6	7	8	9	10
A	MDA-HUVEC EVh				
					

B	MDA-HDFa EVh				
C	MDA-THP1 Vh				
D	HUVEC-HDFa EVh				
E	HUVEC-THP1 EVh				
F	HDFa-THP1 EVh				
G					
H					
		11	12		
A					
B					
C					



3 Apply  70 μL of the fluorescent gelatin working solution at  0.2 mg/mL directly to the bottom of each well.
Avoid the formation of bubbles.

4 Incubate plate for  00:30:00 at  37 °C 5% CO₂.

30m



5 Carefully remove excess coating by aspiration.
Avoid touching well bottom.

6 Pre-condition coating with  200 μL OptiMEM for  00:30:00 at  37 °C 5% CO₂.

30m



Cell Staining and Seeding

30m

7 Subculture cells as usual. Resuspend cell pellets in OptiMEM and count cells using the trypan blue exclusion method.

8 Stain cells using **CellTrace™ CSFE** or **CellTracker™ CMPTX**

8.1 Separate each cell line in two 15-ml conical tubes, each containing 1×10^6 cells in  1 mL OptiMEM.

8.2 Add  1 μL of CellTrace™ CSFE or  0.5 μL of CellTracker™ CMPTX to cell suspensions, to achieve a 5 μM final concentration. Pipette well to mix.



8.3 Incubate cell suspensions at  37 °C 5% CO₂ for  00:30:00 , protected from light.

30m

8.4 Dilute cell suspensions with  4 mL OptiMEM and centrifuge at  1200 rpm, 00:05:00

5m



8.5 Resuspend cell pellets in 1 mL OptiMEM and recount cells using the trypan blue exclusion method.

9 Remove the pre-conditioning media from the plate.

10 Add cell suspensions to wells following the table below:



	A	B	C
	Group	Cell #1 (CellTracker)	Cell #2 (CellTrace)
	MDA-HUVEC	MDA-MB-231 (5000 cells/well)	HUVEC (5000 cells/well)
	MDA-HDFa	MDA-MB-231 (5000 cells/well)	HDFa (2000 cells/well)
	MDA-THP1	MDA-MB-231 (5000 cells/well)	THP-1 (2000 cells/well)
	HUVEC-HDFa	HUVEC (5000 cells/well)	HDFa (2000 cells/well)
	HUVEC-THP1	HUVEC (5000 cells/well)	THP-1 (2000 cells/well)
	HDFa-THP1	HDFa (2000 cells/well)	THP-1 (2000 cells/well)

Note that the total well volume should not surpass 200 µl!

11 Treat cells with EVh (10⁹ particles/ml) or the equivalent treatment volume in PBS.

12 Incubate plate for 24:00:00 at 37 °C 5% CO₂.

1d



Fixation and Cell Staining

10m

13 Remove the supernatant by aspiration.

14 Fix cells with 100 µL warmed 4% PFA at Room temperature for 00:10:00

10m

15 Wash cells twice with 100 µL PBS.



- 16 Permeabilize cells with  100 μ L 0.1% Triton X-100 at  Room temperature for 5m
 00:05:00
- 17 Wash cells twice with  100 μ L PBS.
- 18 Stain cells with the DAPI + Phalloidin-647 mixture. Add  100 μ L of staining solution to each well and incubate at  Room temperature , protected from light for 20m
 00:20:00
- 19 Wash cells twice with  100 μ L PBS.
- 20 Maintain wells with  200 μ L PBS for analysis. Seal the plate with  Parafilm and cover it with aluminum foil for storage at  4 $^{\circ}$ C for up to 6 months.

Cell Imaging by Epifluorescence HTS

- 21 Using the microscope ImageXpress Micro XLS+ (Molecular Devices), check the template for the Corning 3603 plate and the filters for DAPI (nuclei), FITC (gelatin), TxRed (CMPTX) and Cy5 (phalloidin-647). 
- 22 Set laser intensity to a minimum of 10 ms and increase gradatively if necessary. 
- 23 Check the wells using the 4X objective. 
- 24 Change into the 20x objective and adjust the laser focus. Select 9 sites per well minimally. 
- 25 Acquire the plate. Export metadata for analysis. 
- 26 For representative images, change to the 40x objective and adjust the laser focus. Select the sites of interest and acquire. Export image channels and combinations. 



Gelatin Degradation Quantification on FIJI

- 27 On FIJI (ImageJ), import HTD files through BioFormats. 
- 28 Images should already be scaled. If not, adjust scale based on the objective lens used for acquisition. 
- 29 Set measurements to contain "Area", "Standard Deviation", "Shape Descriptor", "Mean grey value", "Perimeter" and "Display label". 
- 30 Concatenate all stacks into a single hyperstack. 
- 31 Split channels and select the FITC stack for analysis. 
- 32 Set a threshold to encompass the degradation spots but not the background. Write down the threshold values. Create a new stack with the binary images. 
- 33 To measure the degraded area, analyze particles with a range of "5-Infinity" and select "Summarize". 
- 34 Save CSV file. The degraded area (in μm^2) per site will be compared between groups in the statistical analysis. 

Cell Counting on FIJI

- 35  go to step #27 and continue until Step #30. 
- 36 Split channels and select the DAPI stack for counting. 
- 37 Set a threshold to contain nuclei. Create a new stack with the binary images. 
- 38 To count cells, analyze particles with a range of "10-infinity" and select "Summarize". 



39 Save CSV file.



Cell Morphology Analysis on FIJI

40  [go to step #27](#) and continue until Step #30.



41 Split channels and select the Cy5 stack for analysis.



42 Duplicate the stack as a guide.



43 Merge the Cy5 stack with the TxRed stack to visually differentiate cell types.



44 Set a threshold to encompass cell cytoplasm. Create a new stack with the binary images.



45 Using the duplicated stack as a guide, section cells using the "pencil" tool with a 3 px thickness.



46 To analyze cell morphology, analyze particles with a range of "10-Infinity" and check "Clear Results".



47 Save CSV file. The cell circularity index of each cell will be compared between groups in the statistical analysis.



Image Processing for Representative Cells

48  [go to step #27](#) and continue until Step #30.



49 Split the channels of the stacks of interest.





- 50 Adjust channel colors using the "Lookup Tables" menu.
- 51 Select the cell of interest in a 200×200 px squared selection.
- 52 Save selections in each channel and the combination of all channels in PNG images.



Protocol references

PACHANE, Bianca Cruz et al. Small Extracellular Vesicles from Hypoxic Triple-Negative Breast Cancer Cells Induce Oxygen-Dependent Cell Invasion. International Journal of Molecular Sciences, [s. l.], v. 23, n. 20, p. 12646, 2022.

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