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Fluorescent Gelatin Degradation Assay to Evaluate EVh Action in TME Cells

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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 four cellular models for the tumor microenvironment - MDA-MB-231 (tumor cell), HUVEC (endothelial cell), HDFa (dermal fibroblast) and THP-1 (monocyte). Adapted from Pachane et al (2022) (PMID: 36293503).

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. 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 ºC, 5% CO_2)
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 °C with warmed PBS following the manufacturer's instructions for a concentration of [M] 5 mg/mL. Aliquot in microtubes and maintain at -20 ºC until time of use.
Before use, thaw gelatin at 37 °C for 00:30:00 . Dilute stock to a [M] 0.2 mg/mL working solution with warmed PBS and maintain at 37 °C until use.

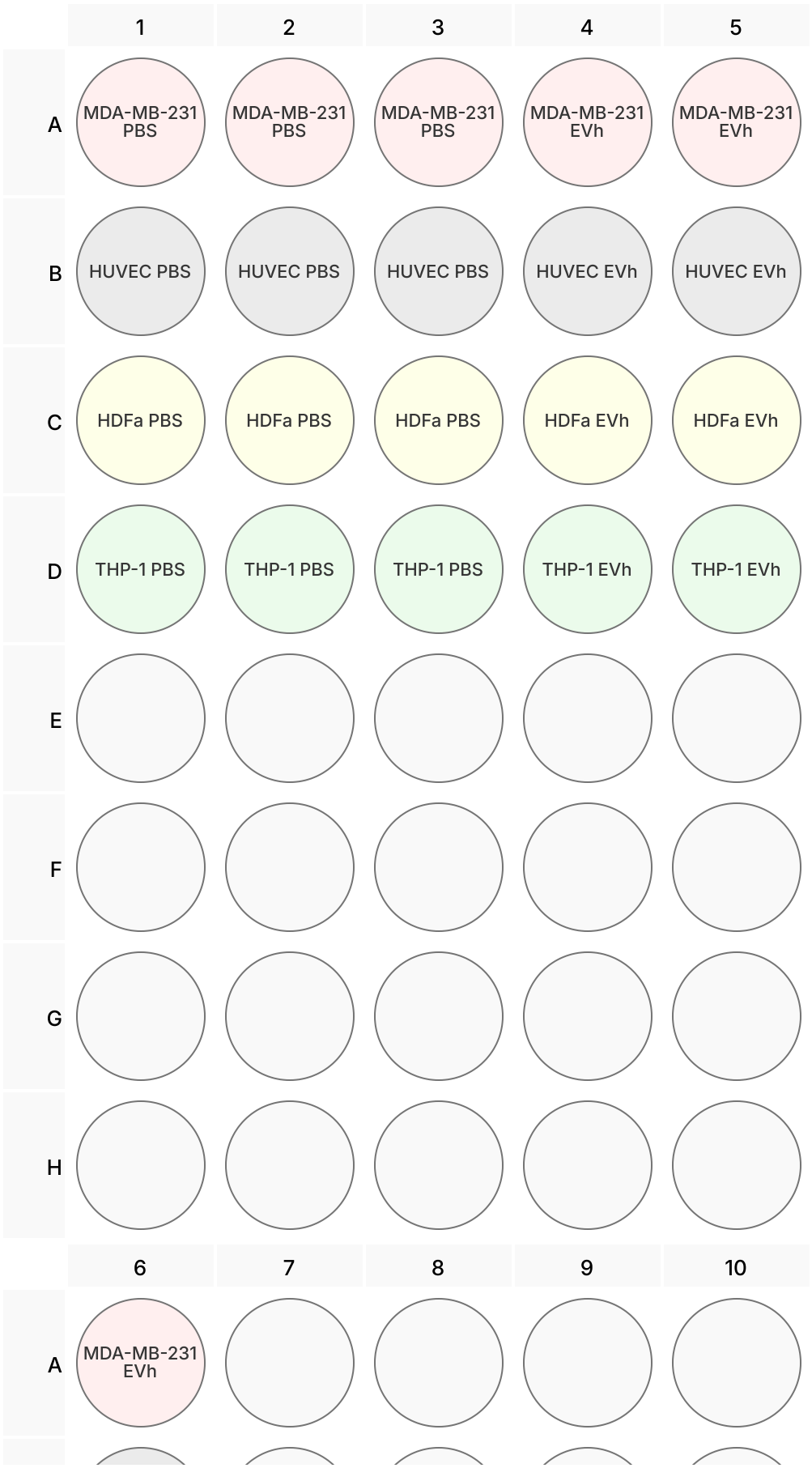
Cell culture: Maintain cells in culture during at least two passages after thawing.

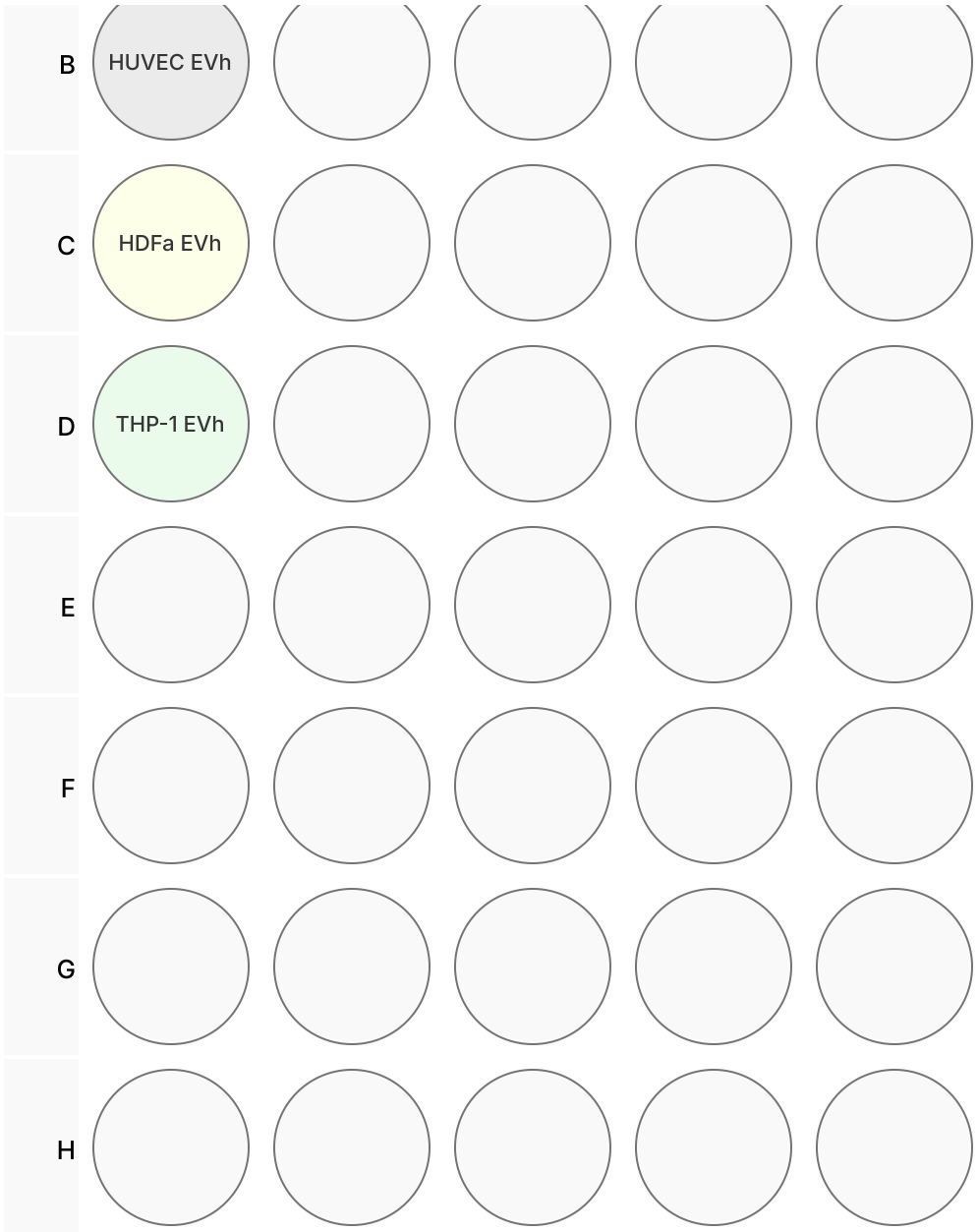
Fluorescent Gelatin Coating

30m

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 cell line.

1.1 Experimental plate map:


1
2
3
4
5
A
MDA-MB-231 PBS
MDA-MB-231 PBS
MDA-MB-231 PBS
MDA-MB-231 EVh
MDA-MB-231 EVh
B
HUVEC PBS
HUVEC PBS
HUVEC PBS
HUVEC EVh
HUVEC EVh
C
HDFa PBS
HDFa PBS
HDFa PBS
HDFa EVh
HDFa EVh
D
THP-1 PBS
THP-1 PBS
THP-1 PBS
THP-1 EVh
THP-1 EVh
E
F
G
H
6
7
8
9
10
A
MDA-MB-231 EVh


B
HUVEC EVh
C
HDFa EVh
D
THP-1 EVh
E
F
G
H
11
12
A
B
C

2 Apply 70 µL of the fluorescent gelatin working solution [M] 0.2 mg/mL directly to the bottom of each well and prevent the formation of bubbles.

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

4 Carefully remove excess coating (avoid touching well bottom).

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

Cell seeding 1d

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

7 Remove pre-conditioning media from the wells (avoid touching well bottom).

8 Add cell suspension into each well to a total volume of 200 µl:

- MDA-MB-231: 5×10^3 cells/well (= 1×10^5 células/ml)
- HUVEC: 5×10^3 cells/well (= 1×10^5 células/ml)
- HDFa: 2×10^3 cells/well (= 1×10^4 células/ml)
- THP-1: 5×10^3 cells/well (= 1×10^5 células/ml)

9 Treat cells with EVh (10^9 particles/ml) or the equivalent treatment volume in PBS.

10 Incubate plate for 24:00:00 at 37 °C 5% CO2 1d

Fixation and Cell Staining 10m

11 Remove the supernatant by aspiration.

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

13 Wash wells twice with 100 µL PBS

14 Permeabilize cells with 100 µL 0.1% Triton X-100 at Room temperature for 00:05:00 5m

15 Wash wells twice with 100 µL PBS

16 Stain cells with the DAPI + Phalloidin-647 mixture. Add 100 µL of staining solution to each well and incubate at Room temperature , protected from light for 00:20:00 . 20m

17 Wash wells twice with 100 µL PBS

18 Maintain wells with 200 µL PBS for analysis. Seal the plate with Parafilm and cover it with aluminum foil for storage at 4 °C for up to 6 months.

Cell Imaging by Epifluorescence HTS

19 Using the microscope **ImageXpress Micro XLS+ (Molecular Devices)**, check the template for the Corning 3603 plate and the filters for DAPI (nuclei), FITC (gelatin) and Cy5 (phalloidin-647).

20 Set laser intensity to a minimum of 10 ms and increase gradatively if necessary.

21 Check the wells using the 4X objective.

22 Change into the 20x objective and adjust the laser focus. Select 9 sites per well minimally.

23 Acquire the plate. Export metadata for analysis.

24 For representative images, change into 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

25 On FIJI (ImageJ), import HTD files through BioFormats.

26 Images should already be scaled. If not, adjust scale based on the objective lens used for acquisition.

27 Set measurements to contain "Area", "Standard Deviation", "Shape Descriptor", "Mean grey value", "Perimeter" and "Display label".

28 Concatenate all stacks into a single hyperstack.

29 Split channels and select the FITC stack for analysis.

30 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.

31 To measure the degraded area, analyze particles with a range of "5-Infinity" and select "Summarize".

32 Save CSV file. The degraded area (in μm^2) per site will be compared between groups in the statistical analysis.

Cell Counting on FIJI

33 go to step #25 and follow through step #27

34 Split channels and select the DAPI stack for counting.

35 Set a threshold to contain nuclei. Create a new stack with the binary images.

36 To count cells, analyze particles with a range of "10-infinity" and select "Summarize".

37 Save CSV file.

Cell Morphology Analysis on FIJI

38 go to step #25 and follow through step #27

39 Split channels and select the Cy5 stack for analysis.

40 Duplicate the stack as a guide.

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

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

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

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

Image Processing for Representative Cells

45 go to step #25 and follow through step #27

46 Split the channels of the stacks of interest.

47 Adjust channel colors using the "Lookup Tables" menu.

48 Select the cell of interest in a 200×200 px squared selection.

49 Save selections in each channel and the combination of all channels in PNG images.

Protocol references

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