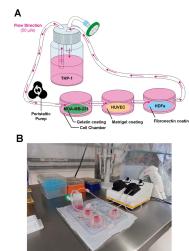


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## Multicellular Circulating Co-Culture

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Wanessa Fernanda Altei: Initial development of method

Heloisa Sobreiro Selistre de Araujo: Financial support and System acquisition



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**Protocol status:** Working

**We use this protocol and it's working**

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## Abstract

A novel method to study the tumor microenvironment (TME) *in vitro*, using the quasi-vivo technology from Kirstall to survey the individual responses in cell types common to the TME. We have developed a strategy that allows a tumoral cell (MDA-MB-231) seeded in gelatin coating, an endothelial cell (HUVEC) seeded in Matrigel coating, and a dermal fibroblast (HDFa) seeded in fibronectin to be cultured *in tandem*, alongside suspension monocytes (THP-1). The goal was to investigate how cells would behave in this setting and evaluate the role of hypoxic tumoral extracellular vesicles in the development of the TME.

## Image Attribution

The diagram was created using Adobe Photoshop. Original photograph by Bianca Pachane.

## Materials

### Materials:

1. Round glass coverslips 13mm Ø
2. 24-well clear plates with flat bottom
3. Histological slides, Exacta.
4. Sterile forceps
5. Petri dishes
6. 0.22 pore syringe filters

### Reagents and Solutions:

1.  Parafilm®; M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher Catalog #1337410**
2.  Poly-L-lysine, 0.1% (wt/vol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P8920**
3.  Glutaraldehyde solution (50% in solution) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G6403**
4.  Gelatin From Pig Skin, Fluorescein Conjugate **Invitrogen - Thermo Fisher Catalog #G13187**
5.  Corning® Matrigel® **Corning Catalog #354277**
6.  Fibronectin **Gibco - Thermo Fisher Catalog #33016-015**
7.  1X PBS (Phosphate-buffered saline )
8.  OptiMEM™ I Reduced Serum Media **Gibco, ThermoFisher Catalog #31985070**
9.  Trypan Blue Solution 0.4% Sterile-filtered **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154**
10.  CellTracker®; Red CMTPX Dye **Thermo Fisher Catalog #C34552** - reconstituted in DMSO
11.  Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P6148** - PFA 4% in deionized water, pH 7.6, sterile
12.  Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8787-50ML** - Triton X-100 0.1% (v/v) in deionized water
13.  Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A7030** - 1% BSA-PBS
14.  Anti-VEGF Receptor 2 antibody **Abcam Catalog #ab39256** - 1:50 dilution in 1% BSA-PBS
15.  BD Transduction Laboratories™ Purified Mouse Anti-β-Catenin **BD Biosciences Catalog #610154** - 1:50 dilution in 1% BSA-PBS
16.  Anti-Collagen II antibody **Abcam Catalog #ab85266** - 1:50 dilution in 1% BSA-PBS
17.  Anti-Collagen III antibody [1E7-D7/Col3] **Abcam Catalog #ab23445** - 1:50 dilution in 1% BSA-PBS
18.  Goat Anti-Mouse IgG H&L (FITC) **Abcam Catalog #ab6785** - 1:1,000 dilution in 1% BSA-PBS
19.  Goat Anti-Rabbit IgG H&L (Alexa Fluor® 568) **Abcam Catalog #ab175696** - 1:1,000 dilution in 1% BSA-PBS

20. Phalloidin + DAPI (1 µl)  Phalloidin-iFluor 647 Reagent Abcam Catalog #ab176759 + 0.76 µl  
     4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) Thermo Fisher Scientific Catalog #D1306 in 5 ml PBS
21.  Fluoromount Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680

**Cell lines and growth media:**

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

**Equipments:**

1. Biological cabinet
2. Cell incubator (37 °C, 5% CO<sub>2</sub>)
3. QV500 System, Kirstall
4. Cell counter - TC20 Cell Counter, Bio-Rad - Catalog #1450011
5. Epifluorescence microscope - ImageXpress Micro XLS, Molecular Devices - Catalog #500496

## Protocol materials

☒ Parafilm®; M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) Thermo Fisher Catalog #1337410

In Materials and 4 steps

☒ Goat Anti-Mouse IgG H&L (FITC) Abcam Catalog #ab6785 Materials, Step 51

☒ Gelatin From Pig Skin, Fluorescein Conjugate Invitrogen - Thermo Fisher Catalog #G13187 Materials

☒ Anti-Collagen II antibody Abcam Catalog #ab85266 Materials, Step 46

☒ Fibronectin Gibco - Thermo Fisher Catalog #33016-015 Materials

☒ Phalloidin-iFluor 647 Reagent Abcam Catalog #ab176759 Materials, Step 56

☒ Poly-L-lysine, 0.1% (wt/vol) Merck MilliporeSigma (Sigma-Aldrich) Catalog #P8920 Materials, Step 3

☒ Glutaraldehyde solution (50% in solution) Merck MilliporeSigma (Sigma-Aldrich) Catalog #G6403

Materials, Step 2

☒ Paraformaldehyde Merck MilliporeSigma (Sigma-Aldrich) Catalog #P6148 Materials

☒ BD Transduction Laboratories™ Purified Mouse Anti-β-Catenin BD Biosciences Catalog #610154

Materials, Step 46

☒ Bovine Serum Albumin Merck MilliporeSigma (Sigma-Aldrich) Catalog #A7030 Materials

☒ 4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) Thermo Fisher Scientific Catalog #D1306 Materials, Step 56

☒ OptiMEM™ I Reduced Serum Media Gibco, ThermoFisher Catalog #31985070 In Materials and 7 steps

☒ Trypan Blue Solution 0.4% Sterile-filtered Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154

In Materials and 3 steps

☒ Fluoromount Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680 Materials, Step 58

☒ Corning® Matrigel® Corning Catalog #354277 Materials

☒ Triton X-100 Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8787-50ML Materials

☒ CellTracker® Red CMTPX Dye Thermo Fisher Catalog #C34552 Materials, Step 22

☒ Anti-Collagen III antibody [1E7-D7/Col3] Abcam Catalog #ab23445 Materials, Step 46

☒ Anti-VEGF Receptor 2 antibody Abcam Catalog #ab39256 Materials, Step 46

☒ Goat Anti-Rabbit IgG H&L (Alexa Fluor® 568) Abcam Catalog #ab175696 Materials, Step 51

## Safety warnings

❗ Light-sensitive assay. Work under sterile conditions.

## Before start

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

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

**Matrigel preparation:** Thaw Matrigel vial at  $4 \text{ }^{\circ}\text{C}$  and maintain  $\text{On ice}$  until use. Dilute a 1:1, v/v aliquot of stock Matrigel in cold sterile PBS for use.

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

**QV500:** Autoclave all parts before use.

## Preparation of matrix-coated coverslips

20m

1 Clean round glass coverslips (13 mm ø) with 70% ethanol wipes before use. Maintain slips in a clean container.

2 Prepare a 0.5% solution of glutaraldehyde in H<sub>2</sub>O and maintain it at  4 °C protected from light.

 Glutaraldehyde solution (50% in solution) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G6403**

3 Under sterile conditions, prepare a surface with a piece of Parafilm fixed in place. Add  20 µL droplets of poly-L-lysine interspaced in the Parafilm.

 Poly-L-lysine, 0.1% (wt/vol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P8920**

 Parafilm® M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher Catalog #1337410**

4 Drop coverslips atop the droplets and incubate at  Room temperature for  00:20:00 minimum.

20m



5 Using forceps, transfer the coverslips to a 24-well plate with the coating facing upwards.

6 Wash coverslips twice with  500 µL PBS.

 1X PBS (Phosphate-buffered saline )

7 Cross-link coating with  500 µL of cold  0.5 % (v/v) glutaraldehyde for  00:15:00 at  Room temperature

15m



8 Prepare a Petri dish with the bottom covered in Parafilm.

 Parafilm® M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher Catalog #1337410**

9 Apply spaced  20 µL droplets of the matrices to the Parafilm-covered surface:

- **Fluorescent gelatin:**  0.2 mg/mL in PBS, kept at  37 °C until time of use;
- **Matrikel solution:**  50 % (v/v) in PBS, kept  On ice until time of use;
- **Fibronectin solution:**  1 mg/mL in PBS, kept  On ice until time of use;



10 Remove the coverslips from the 24-well plate and drop them atop the droplets, with the coating facing down. Incubate at 4 °C Overnight, protected from light.



11 The next day, remove the slips from the Petri dish using a forceps and transfer them, with the coating facing up, to a fresh 24-well plate.

12 Wash coverslips twice with 500 µL PBS.

1X PBS (Phosphate-buffered saline )

13 *Slips can be stored at 4 °C for up to a week, wrapped in aluminium foil.*



14 Pre-condition matrix coating with 500 µL of culture media for 00:30:00 at

37 °C :

- **Fluorescent gelatin:** Leibovitz L-15 10% FBS
- **Matrigel solution:** DMEM 10% FBS 1% pen/strep
- **Fibronectin solution:** DMEM 10% FBS 1% pen/strep

30m



## Cell seeding

15 Subculture cells as usual. Resuspend cell pellets in growth media and count cells using the trypan blue exclusion method.

- **MDA-MB-231** - Leibovitz L-15 10% FBS
- **HUVEC** - DMEM 10% FBS 1% pen/strep
- **HDFa** - DMEM 10% FBS 1% pen/strep

Trypan Blue Solution 0.4% Sterile-filtered **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154**

16 Remove the pre-conditioning medium from the 24-well plate.

17 Seed cells in the 24-wells following the table below:

A	B	C	D
<b>Coating:</b>	<b>Cell Type:</b>	<b>Density:</b>	<b>Culture Media:</b>
Fluorescent gelatin	MDA-MB-231	50,000 cells/ml	1 ml Leibovitz L-15 10% FBS
Matrigel	HUVEC	50,000 cells/ml	1 ml DMEM 10% FBS 1% pen/strep
Fibronectin	HDFa	20,000 cells/ml	1 ml DMEM 10% FBS 1% pen/strep

- 18 Incubate cells at  37 °C 5% CO<sub>2</sub>  Overnight for adhesion.  
*ps: seal the MDA-MB-231 plate with parafilm to protect from CO<sub>2</sub> exposure.*



## THP-1 staining and seeding

5m

- 19 The day after cell seeding:  
Transfer THP-1 suspension to a conical tube and centrifuge at  1200 rpm, 00:05:00 .  
Discard supernatant.
- 20 Resuspend cell pellet in OptiMEM 1% pen/strep and count cells using the trypan blue exclusion method.  
 OptiMEM™ I Reduced Serum Media **Gibco, ThermoFisher Catalog #31985070**  
 Trypan Blue Solution 0.4% Sterile-filtered **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154**
- 21 Dilute cell suspension to a  $1 \times 10^6$  cells/ml density in OptiMEM 1% pen/strep.  
 OptiMEM™ I Reduced Serum Media **Gibco, ThermoFisher Catalog #31985070**
- 22 Add  1  $\mu$ L of CellTracker™ CMPTX per ml of cell suspension for a  $5 \text{ micromolar } (\mu\text{M})$  final concentration. Pipette well to mix.  
 CellTracker® Red CMTPX Dye **Thermo Fisher Catalog #C34552**
- 23 Incubate the cell suspension at  37 °C % CO<sub>2</sub> for  00:30:00 , protected from light.
- 30m
- 24 Dilute cell suspensions with  4 mL OptiMEM and centrifuge at  1200 rpm, 00:05:00  
 OptiMEM™ I Reduced Serum Media **Gibco, ThermoFisher Catalog #31985070**
- 25 Resuspend cell pellets in  1 mL OptiMEM and recount cells using the trypan blue exclusion method.  
 OptiMEM™ I Reduced Serum Media **Gibco, ThermoFisher Catalog #31985070**  
 Trypan Blue Solution 0.4% Sterile-filtered **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154**
- 26 In a 15-ml conical tube, prepare a  $1 \times 10^5$  cell/ml THP-1 suspension in  15 mL OptiMEM 1% pen/strep . Maintain protected from light.  
 OptiMEM™ I Reduced Serum Media **Gibco, ThermoFisher Catalog #31985070**

## QV500 assembly and preparation

10m

27 QV500 consists of:

- (1) Peristaltic pump
- (3) Culture chambers with connecting tubings
- (1) 15-ml reservoir with 1 inlet, 1 outlet and 1 air filter piece.
- (1) 1.6 mm pump tubing
- (2) plastic chamber supports

*Check the experimental diagram for better understanding.*

28 Assemble the system under the biological hood with autoclaved parts:

28.1 Add  15 mL sterile PBS to the reservoir and close the lid. 1X PBS (Phosphate-buffered saline )

28.2 Attach the 0.22 µm pore syringe filter to the air outlet (blue piece).

28.3 Add  1 mL sterile PBS to each chamber and close the lids. 1X PBS (Phosphate-buffered saline )

28.4 Attach the 1.6 mm pump tubing to the peristaltic pump.

28.5 Connect the inlet and outlet tubings with the chambers to form a close circuit.

28.6 Circulate the PBS for  00:10:00 at max flow rate.

10m



29 Drain the system and disassemble the parts.

30 Repeat steps  go to step #28.1 until step #29 with 15 ml OptiMEM to condition the system. OptiMEM™ I Reduced Serum Media **Gibco, ThermoFisher Catalog #31985070**

## Multicellular Circulating Co-Culture

1d

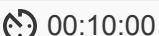
- 31 Remove the growth media from cells seeded in coverslips (  go to step #18 ).
- 32 Assemble the MDA-gelatin coverslip in one QV500 culture chamber; the HUVEC-Matrigel coverslip in the second culture chamber; and the HDFa-FN coverslip in the last culture chamber.  

- 33 Add  1 mL 1 ml OptiMEM 1% pen/strep to each chamber and close the lids, connecting the tubings following the order described above.  
 OptiMEM™ I Reduced Serum Media **Gibco, ThermoFisher Catalog #31985070**
- 34 Add the  15 mL THP-1 cell suspension in OptiMEM 1% pen/strep to the reservoir.
- 35 Add EVh ( $10^9$  particles/ml) or the equivalent treatment volume in PBS to the reservoir and close the lid.
- 36 Connect the tubings to close the system. Circulate the media at a 50  $\mu$ l/s flow rate and incubate the whole system for  24:00:00 at  37 °C 5% CO<sub>2</sub>.  
  

- 37 The next day, disconnect the system and carefully drain the compartments. Collect the conditioned media in a conical tube and spin at  1200 rpm, 4°C, 00:10:00. Transfer the supernatant to fresh tubes and freeze at  -80 °C for further testing.  
  

- 38 Transfer the coverslips to a fresh 24-well plate for fixing and staining (  go to step #40 )
- 39 Wash and decontaminate the QV500 parts for further use.

## Cell Fixing

- 40 Fix cells in coverslips with  500  $\mu$ L warmed 4% PFA at  Room temperature for  00:10:00  

- 41 Wash coverslips twice with  500  $\mu$ L PBS.

 1X PBS (Phosphate-buffered saline )

42 Permeabilize cells with  500 µL 0.1% Triton X-100 at  Room temperature for  00:05:00 

43 Wash coverslips twice with  500 µL PBS.

 1X PBS (Phosphate-buffered saline )

44 For **MDA-MB-231 coverslips only:** skip the immunofluorescence protocol and  go to step #56 

## Immunofluorescence of HUVEC and HDFa Coverslips

45 Block non-specific bindings with  500 µL 1% BSA-PBS for  01:00:00 at  4 °C 

46 Prepare the primary antibody dilutions: 

### For HUVECs:

1.  Anti-VEGF Receptor 2 antibody **Abcam Catalog #ab39256** - 1:50 dilution in 1% BSA-PBS
2.  BD Transduction Laboratories™ Purified Mouse Anti-β-Catenin **BD Biosciences Catalog #610154**  
- 1:50 dilution in 1% BSA-PBS

### For HDFa:

1.  Anti-Collagen II antibody **Abcam Catalog #ab85266** - 1:50 dilution in 1% BSA-PBS
2.  Anti-Collagen III antibody [1E7-D7/Col3] **Abcam Catalog #ab23445** - 1:50 dilution in 1% BSA-PBS

47 Place  20 µL of spaced droplets of the antibodies in a Petri dish covered with Parafilm (as seen on  go to step #8 ).

 Parafilm&trade; M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher Catalog #1337410**

48 Remove the coverslips from the 24-well plate and drop them atop the droplets, with the cells facing down. Incubate at  4 °C for  18:00:00 , protected from light. 

49 The next day, remove the slips from the Petri dish using a forceps and transfer them, with the coating facing up, to a fresh 24-well plate.

50 Wash coverslips twice with  500 µL PBS.

 1X PBS (Phosphate-buffered saline )

51 Prepare the secondary antibody dilutions:

1.  Goat Anti-Mouse IgG H&L (FITC) **Abcam Catalog #ab6785** - 1:1,000 dilution in 1% BSA-PBS

2.  Goat Anti-Rabbit IgG H&L (Alexa Fluor® 568) **Abcam Catalog #ab175696** - 1:1,000 dilution in 1% BSA-PBS

52 Place  20 µL of spaced droplets of the antibodies in a Petri dish covered with Parafilm (as seen on  go to step #8 ).

 Parafilm® M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher Catalog #1337410**

53 Remove the coverslips from the 24-well plate and drop them atop the droplets, with the cells facing down. Incubate at  Room temperature for  01:00:00 , protected from light.

1h



54 Remove the slips from the Petri dish using a forceps and transfer them, with the coating facing up, to a fresh 24-well plate.

55 Wash coverslips twice with  500 µL PBS.

 1X PBS (Phosphate-buffered saline )

## Cell Counterstaining and Slide Assembly

20m

56 Add  500 µL of **DAPI + Phalloidin-647 mixture** to each well and incubate for  00:20:00 .

20m



> 1 µl  Phalloidin-iFluor 647 Reagent **Abcam Catalog #ab176759** + 0.76 µl

 4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #D1306**

in 5 ml PBS

57 Wash coverslips twice with  500 µL PBS.

### 1X PBS (Phosphate-buffered saline )

- 58 Remove the coverslips from the 24-well plate and assemble them in histological slides with mounting media. Allow the media to dry for at least  04:00:00 .

 Fluoromount Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680

4h



- 59 Once dry, seal coverslips using clear nail polish and store at  4 °C until time of analysis.

II

## Cell Imaging by Epifluorescence HTS

- 60 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).

- 61 Set laser intensity to a minimum of 10 ms and increase gradually if necessary.

- 62 Check the wells using the 4X objective.

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

- 64 Acquire the plate. Export metadata for analysis.

- 65 Repeat  go to step #63 and step #64 with the 40x objective.

- 66 For representative images, use the 40x objective and adjust the laser focus.

Select the sites of interest and acquire.

Export image channels and combinations.

## Imaging analysis

- 67 Gelatin degradation quantification, cell morphology analysis, quantification of immunofluorescence probing, and assembly of representative images were performed using FIJI.

## Software

ImageJ/Fiji

NAME

Windows 7

OS

National Institutes of Health

DEVELOPER

<http://wsr.imagej.net/distros/win/ij152-win-java8.zip>

SOURCE LINK

Please refer to the following protocol for the complete pipeline of analysis.

## Protocol



NAME

Cell Invasion in Direct Co-Culture

CREATED BY

Bianca Cruz Pachane

[PREVIEW](#)

## 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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