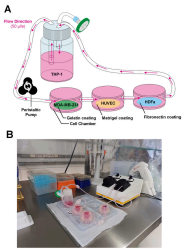
Sep 10, 2024

Multicellular Circulating Co-Culture



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Wanessa Fernanda Altei: Initial development of method
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Protocol status: Working
We use this protocol and it's working

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Disclaimer

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 Scientific Catalog #**33016-015
7. 1X PBS (Phosphate-buffered saline)
8. OptiMEM™ I Reduced Serum Media **Gibco - Thermo Fisher Scientific 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 ºC, 5% CO_2)
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

- 1X PBS (Phosphate-buffered saline)
- Parafilm™ M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher Catalog #**1337410
- CellTracker™ Red CMTPX Dye **Thermo Fisher Catalog #**C34552
- OptiMEM™ I Reduced Serum Media **Gibco - Thermo Fisher Scientific Catalog #**31985070
- Trypan Blue Solution 0.4% Sterile-filtered **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8154
- Poly-l-lysine, 0.1% (wt/vol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P8920
- Glutaraldehyde solution (50% in solution) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G6403
- Anti-Collagen III antibody [1E7-D7/Col3] **Abcam Catalog #**ab23445
- Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P6148
- BD Transduction Laboratories™ Purified Mouse Anti-β-Catenin **BD Biosciences Catalog #**610154
- Goat Anti-Mouse IgG H&L (FITC) **Abcam Catalog #**ab6785
- Fluoromount **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F4680
- Gelatin From Pig Skin, Fluorescein Conjugate **Invitrogen - Thermo Fisher Catalog #**G13187
- Corning® Matrigel® **Corning Catalog #**354277
- Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A7030
- Anti-VEGF Receptor 2 antibody **Abcam Catalog #**ab39256
- Anti-Collagen II antibody **Abcam Catalog #**ab85266
- Goat Anti-Rabbit IgG H&L (Alexa Fluor® 568) **Abcam Catalog #**ab175696
- 4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #**D1306
- Fibronectin **Gibco - Thermo Fisher Scientific Catalog #**33016-015
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787-50ML
- Phalloidin-iFluor 647 Reagent **Abcam Catalog #**ab176759

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 °C 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.

Matrigel preparation: Thaw Matrigel vial at 4 °C and maintain 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_2O 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 [M] 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:** [M] 0.2 mg/mL in PBS, kept at 37 °C until time of use;
- **Matrigel solution:** [M] 50 % (v/v) in PBS, kept On ice until time of use;
- **Fibronectin solution:** [M] 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 ºC 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 : 30m

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

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
	Coating:	**Cell Type:**	**Density:**	**Culture Media:**
	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

	A	B	C	D
	Fibronectin	HDFa	20,000 cells/ml	1 ml DMEM 10% FBS 1% pen/strep

18 Incubate cells at 37 °C 5% CO2 Overnight for adhesion.

ps: seal the MDA-MB-231 plate with parafilm to protect from CO_2 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. 5m

20 Resuspend cell pellet in OptiMEM 1% pen/strep and count cells using the trypan blue exclusion method.

OptiMEM™ I Reduced Serum Media **Gibco - Thermo Fisher Scientific Catalog #**31985070

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

21 Dilute cell suspension to a 1×10^6 cells/ml density in OptiMEM 1% pen/strep.

OptiMEM™ I Reduced Serum Media **Gibco - Thermo Fisher Scientific Catalog #**31985070

22 Add 1 µL of CellTracker™ CMPTX per ml of cell suspension for a [M] 5 micromolar (µM) final concentration. Pipette well to mix.

CellTracker™ Red CMTPX Dye **Thermo Fisher Catalog #**C34552

23 Incubate the cell suspension at 37 °C % CO2 for 00:30:00 , protected from light. 30m

24 Dilute cell suspensions with 4 mL OptiMEM and centrifuge at 1200 rpm, 00:05:00 5m

OptiMEM™ I Reduced Serum Media **Gibco - Thermo Fisher Scientific 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 - Thermo Fisher Scientific Catalog #**31985070

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

26 In a 15-ml conical tube, prepare a 1×10^5 cell/ml THP-1 suspension in 15 mL OptiMEM 1% pen/strep. Maintain protected from light.

OptiMEM™ I Reduced Serum Media **Gibco - Thermo Fisher Scientific 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 - Thermo Fisher Scientific 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 - Thermo Fisher Scientific 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 µl/s flow rate and incubate the whole system for 24:00:00 at 37 °C 5% CO2.

1d

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.

10m

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 µL warmed 4% PFA at Room temperature for 00:10:00 10m

41 Wash coverslips twice with 500 µL PBS.
1X PBS (Phosphate-buffered saline)

42 Permeabilize cells with 500 µL 0.1% Triton X-100 at Room temperature for 00:05:00 5m

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 1h

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™ 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.

18h

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 . 4h
Fluoromount **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F4680

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

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 gradatively 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	REPOSITORY
https://imagej.nih.gov/ij/	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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