NCL Method IEA-1

Analysis of Nanoparticle Effects on Invasion of Cancer Cells

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

This document provides a protocol for a real-time, quantitative assessment of nanoparticle-mediated inhibition of cancer cell invasion. Although processes of cell migration and invasion have similar properties, they can be differentiated based on the ability of invasive cells to migrate through either an extracellular matrix (ECM) or a basement membrane extract (BME). This protocol uses Matrigel as an extracellular matrix and analyzes the number of cells migrating through the Matrigel and porous membrane barriers with or without prior exposure to test nanoparticles. Nanoparticles, which intended mechanism of action involves the inhibition of cancer cell invasion, will result in a decrease in the number of cells migrated through these barriers.

2. Principles

This assay uses human breast cancer cells, MDA-MB-231, as a model cell line. The cells are separated from control chemoattractant and test-nanoparticles by an 8 μm filter and an extracellular matrix. The cell migration through the filter is then monitored using a label-free technology developed by ACEA Biosciences, wherein cell attachment to gold electrodes on the underside of the filter results in a change in the impedance. This is subsequently converted into the cell index, which is proportional to the number of cells migrated through the filter. Schematics and pictures showing the migration plate are provided in Appendix 11.1-11.3. While this protocol can be adapted to other cancer cell lines, additional experiments to evaluate optimal plating density would be needed.

3. Reagents, Materials, Cell Lines, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

3.1.1 Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)

3.1.2 Fetal bovine serum (FBS) (GE Life Sciences, Hyclone, SH30070.03)

3.1.3 RPMI-1640 (GE Life Sciences, Hyclone, SH30096.01)

3.1.4 0.25% Trypsin-EDTA (Invitrogen, 25200-056)

3.1.5 AOPI, 5 mL (Nexelcom, CS2-0106-5ML)

3.1.6 L-glutamine (Hyclone, SH30034.01)

3.1.7 Matrigel (Corning, 354234)

3.2 Materials

3.2.1 Pipettes covering the range of 0.05 to 10 mL

3.2.2 RTCA CIM-Plate 16 (ACEA/Agilent, 05665825001)

3.2.3 Polypropylene tubes 50 and 15 mL

3.2.4 Multichannel pipettor

3.2.5 Counting Chamber (Nexelcom, CHT4-SD100-014)

3.2.6 Eppendorf tubes, 1 mL

3.3 Cell Line

3.3.1 MDA-MB-231, (ATCC, ATCC® HTB26™)

3.4 Equipment

3.4.1 Centrifuge capable of operating at 400xg

3.4.2 Refrigerator, 2-8ºC

3.4.3 Freezer, -20ºC

3.4.4 Cell culture incubator with 5% CO_2 and 95% humidity

3.4.5 Biohazard safety cabinet approved for level II handling of biological material

3.4.6 Inverted microscope

3.4.7 Vortex

3.4.8 Cellometer Auto 2000 Cell Counter, (Nexelcom)

3.4.9 xCELLigence® RTCA DP Instrument (ACEA/Agilent, 00380601050)

4. Reagent and Control Preparation

4.1 Complete RPMI-1640 medium

The complete RPMI medium should contain the following reagents:

10% FBS (heat inactivated)

4 mM L-glutamine

Store at 2-8ºC protected from light for no longer than 1 month. Before use, warm in a water bath.

4.2 Starvation Media (SM)

The starvation RPMI medium should contain the following reagents:

1% FBS

4 mM L-glutamine

Store at 2-8°C protected from light for no longer than 1 month. Before use, warm the medium in a water bath.

4.3 Heat-inactivated fetal bovine serum

Thaw a bottle of FBS at room temperature, or overnight at 2-8ºC and allow to equilibrate to room temperature. Incubate 30 minutes at 56ºC in a water bath mixing every 5 minutes. Single-use aliquots may be stored at 2-8ºC for up to one month or at a nominal temperature of -20ºC indefinitely.

4.4 Negative Control

Use starvation medium as a negative control. Process this control the same way as the study samples.

4.5 Positive Control, Complete RPMI-1640 medium

Use growth medium as a positive control. Process this control the same way as the study samples.

5. Preparation of Study Samples

This assay requires 2.4 mL of nanoparticles, at 1X the highest final tested concentration dissolved/resuspended in starvation medium. The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called “theoretical plasma concentration”. Considerations for

estimating theoretical plasma concentration were reviewed elsewhere [1] and are summarized in Box 1 below.

The assay will evaluate 4 concentrations: 10 X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration and two 1:5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration. For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 2 mg/mL will be prepared and diluted 10-fold (0.2 mg/mL), followed by two 1:5 serial dilutions (0.04 and 0.008 mg/mL). Use 160 µL of each of these samples per well. Each nanoparticle concentration is plated 3 times.

Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests

In this example, we assume a mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$Human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\ mg/kg}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in blood, which is used as the in vitro test concentration.

$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

6. MDA-MB-231 Cell Preparation

MDA-MB-231 is a human breast cancer cell line derived from a metastatic site. Cultures can be maintained by subculture at a ratio of 1:5 to 1:10. Do not allow cells to become confluent. Cell morphology should have a flat, epithelial appearance, if grown under optimal conditions.

6.1 Expand cells until they are approximately 60-70% confluent. The day before the experiment, change the medium from normal growth medium to starvation medium and incubate overnight (16 – 18 hr).

6.2 On the day of the experiment, count cells using AOPI and adjust concentration to $4x10^5$ **viable** cells/mL in Starvation Medium.

7. Matrigel Coating of the Upper Chamber of CIM-Plate 16

The day prior to the experiment, place pipette tips, Eppendorf tubes, and the upper chamber of the CIM-Plate 16 at 4°C to cool.

7.1 Dilute the Matrigel with pre-cooled SFM on ice in pre-cooled Eppendorf tubes. Approximately 1 mL of diluted Matrigel is needed to coat all 16 wells of the upper chamber of a CIM-Plate 16.

7.2 Dilute Matrigel with cold SFM to a concentration of 800 µg/mL, being careful to maintain the Matrigel solution on ice to avoid polymerization.

7.3 Add 50 µL of Matrigel solution into each well of the upper chamber. Gently tap the plate the ensure the Matrigel evenly covers the entire surface of each well.

7.4 Remove 30 µL of the nascent Matrigel solution from each well, leaving the remaining 20 µL to coat the membrane surface of each well.
Note: When removing the 30 µL of Matrigel, insert pipette tip into the well as far as possible without touching the membrane; withdraw the Matrigel slowly. Be careful to not introduce air bubbles during this step.

7.5 Place upper chamber in 37°C incubator for 4 hours.

8. Experimental Procedure

The procedure described below is based on reference 2.

8.1 Add 160 µL of growth medium in the bottom chamber of the CIM-Plate 16 (a meniscus should be formed at the top of each well, see figures in Appendix 11.3).

8.2 Attach the Matrigel-coated upper chamber of the CIM-Plate 16, carefully to avoid bubbles.

8.3 Add 30 µL of starvation medium to all 16 wells of the top chamber.

8.4 Place the chamber(s) into the RTCA DP instrument according to the experimental plan and allow medium/test reagents to equilibrate for 60 minutes.

8.5 Perform a background read.

8.6 Remove the plate from the instrument and add 100 µL of the cell suspension to the top wells.

8.7 Allow the cells to settle in the wells at RT for 30 minutes before replacing on the instrument.

8.8 Place chamber(s) in the instrument and start the protocol (see instrument settings in Appendix 11.4).

8.9 Acquire data for 24-48 hours.

9. Calculations

9.1 Calculate area under the curve (AUC) for the control and test samples using instrument software or Excel then compare the AUC of the test samples to that of the control sample. Use statistical analysis to evaluate the significance of the observed difference.

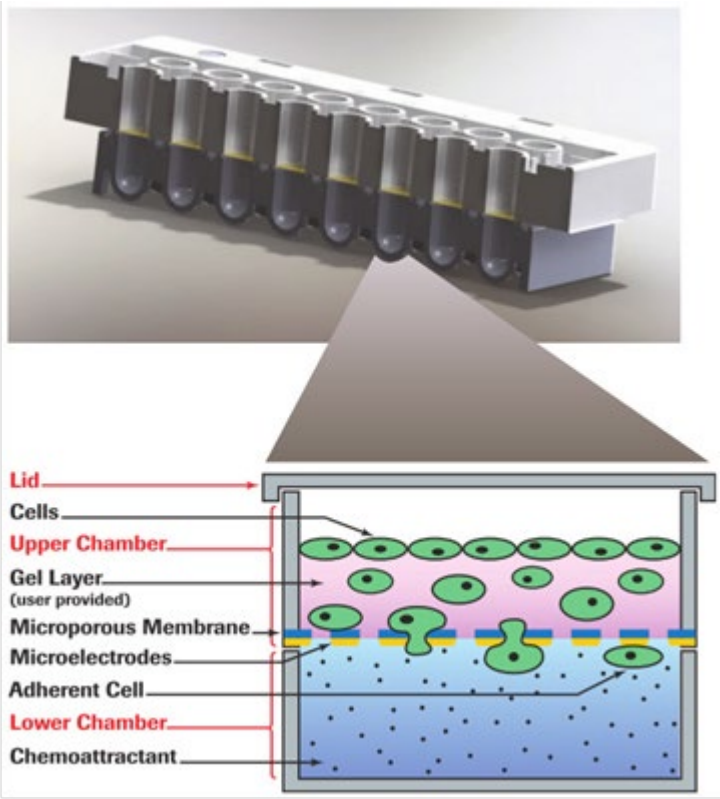
10. References

1. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release. 2013;172(2):456-66.
2. xCELLigence® Real-Time Cell Analysis (CIM Protocol). Using the xCELLigence® RTCA DP Instrument to perform Cell Invasion and Migration (CIM) Assays, ACEA Biosciences 2015
3. Corning Life Sciences. Cell Migration, Chemotaxis and Invasion Assay Protocol. Corning Incorporated.

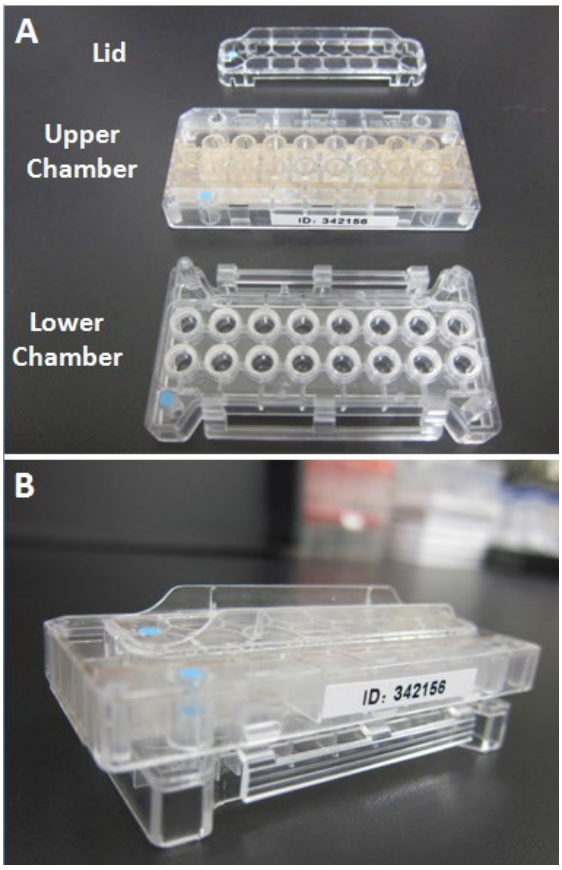
11. Appendix

Images shown in Figures 11.1-11.3 are adopted from reference 2 with permission.

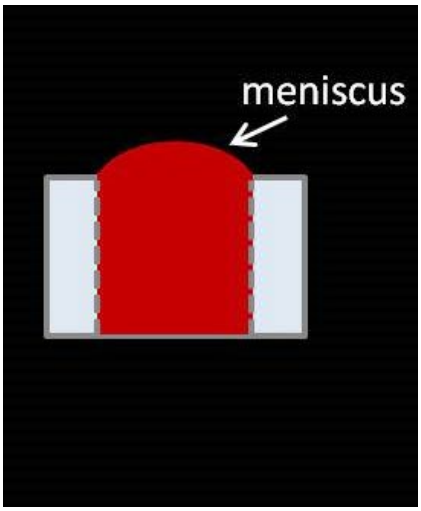
11.1 CIM-Plate 16 Schematic



11.2 ACEA CIM Plate 16 images. Figure A is the ACEA CIM-Plate 16 unassembled. Figure B is the ACEA CIM-Plate 16 assembled.



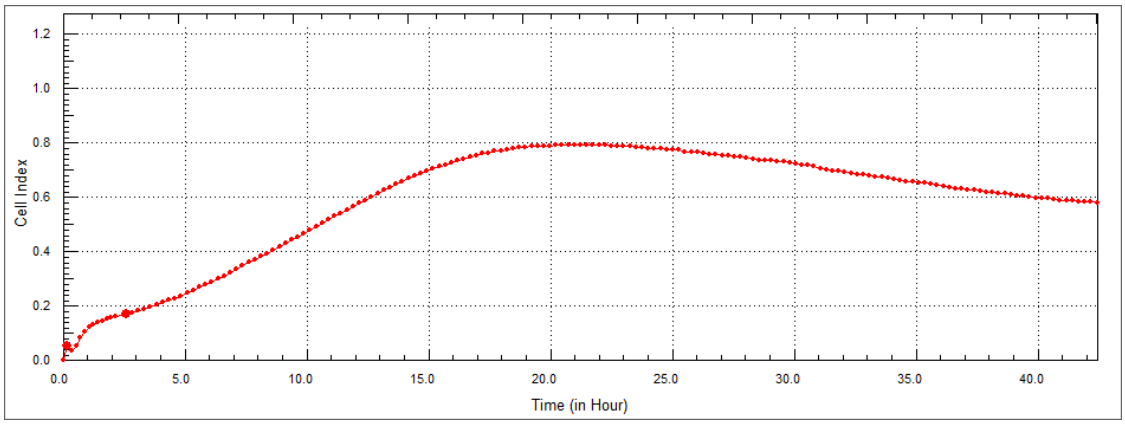
11.3 Reagent loading image, plate bottom

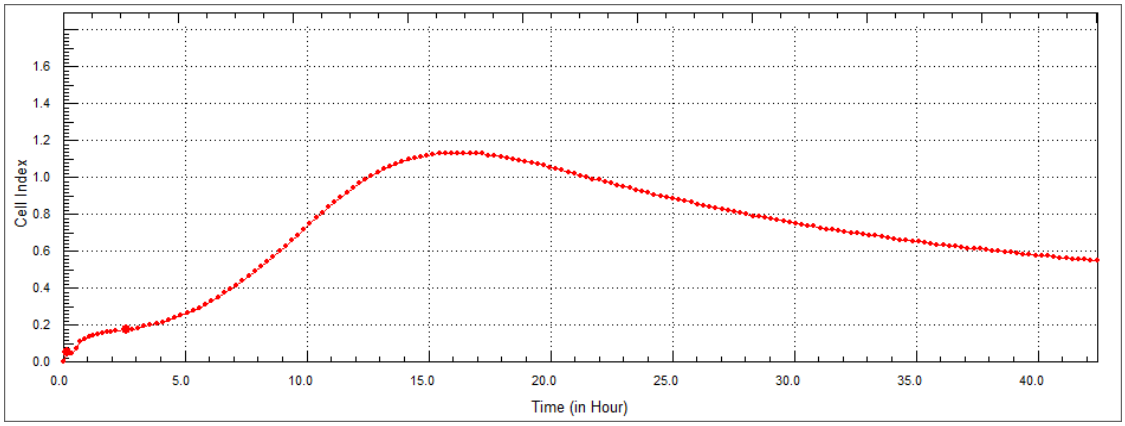


11.4 Instrument settings

Step #	Step Name	Interval (min)	Sweeps
1	Background	1.00	1
2	Cell Attachment	0.50	250
3	Cell Growth	15.0	300

11.5 Example Graphs





12. Abbreviations

CIM	cell invasion/migration
CV	coefficient of variation
FBS	fetal bovine serum
PBS	phosphate buffered saline
RTCA	Real-Time Cell Analysis
SD	standard deviation
SM	starvation media
VC	vehicle control