NCL Method ITA-8.2

Analysis of Nanoparticle Chemoattractant Properties Using Label-Free, Real-Time Chemotaxis Assay

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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 the chemoattractant capacity of engineered nanomaterials. Leukocyte recruitment is a central component of the inflammatory process in both host defense against pathogens and a range of inflammatory disorders. Leukocytes respond to pro-inflammatory stimuli, including but not limited to cytokines, chemokines, and prostaglandins, by migrating from the bloodstream to the site of inflammation.

2. Principles

This assay uses the mouse macrophage cell line RAW 264.7 as a model. The cells are separated from control chemoattractant and test-nanoparticles by an 8 μm filter. Cell migration through the filter is monitored using a label-free technology developed by ACEA Biosciences/Agilent, wherein cell attachment to gold electrodes on the underside of the filter results in a change of impedance, which subsequently is converted into a cell index which is proportional to the number of cells that migrated through the filter (See Appendix 13.1).

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 Bovine serum albumin (BSA) (Sigma-Aldrich, A4503)

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

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

3.1.5 Pen/Strep solution (GE Life Sciences, Hyclone, SV30010)

3.1.6 0.25% Trypsin-EDTA (Invitrogen, 25200-056)

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

3.1.8 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

3.1.9 Fibronectin (Sigma-Aldrich, F2006)

3.1.10 fMLP (Sigma-Aldrich, F3506)

3.1.11 β-mercaptoethanol (Sigma-Aldrich, M3148)

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

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

3.3 Cell Line

3.3.1 RAW 264.7 (mouse macrophage cells), (ATCC, ATCC®-TIB-71™)

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 <u>Complete RPMI-1640 Medium</u>

The complete RPMI medium should contain the following reagents:

10% FBS (heat inactivated)

4 mM L-glutamine

100 U/mL penicillin

100 μg/mL streptomycin sulfate

50 μM β-mercaptoethanol

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:

0.2 % BSA

4 mM L-glutamine

100 U/mL penicillin

100 μg/mL streptomycin sulfate

50 μM β-mercaptoethanol

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 Fibronectin

Reconstitute to 1 mg/mL in cell culture water, then incubate at 37°C for 30 minutes; aliquot 25 μL/vial and store at -20°C. Use 5 μg/mL final concentration for plate coating.

4.5 Negative Control

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

4.6 Positive Control, fMLP (Sigma, F3506)

Reconstitute at 4 mg/mL with DMSO; aliquot 50 μL/vial and store at -20°C. Use 1 ng/mL final concentration.

4.8 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin.

However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

5. Preparation of Study Samples

This assay requires 2.0 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 the “theoretical plasma concentration”. Considerations for estimating theoretical plasma concentration were reviewed elsewhere [1] and are summarized in Box 1 below.

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:

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

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.

$$\text{in vitro concentration}_{\text{human matrix}} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70\ \text{kg x } 10\ \text{mg/kg}}{5.6\ \text{L}} = 0.125\ \text{mg/mL}$$

The assay will evaluate 4 concentrations: 10X (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 three times.

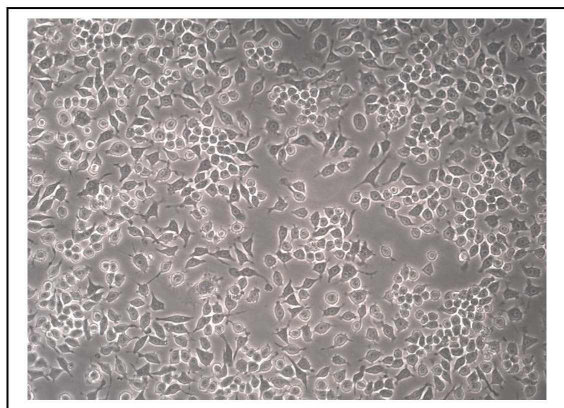
6. RAW 264.7 Cell Preparation

RAW 264.7 is a mouse macrophage cell line. Cultures can be maintained by subculture at a ratio of 1:3 to 1:6. Do not allow cells to become confluent. Cell morphology should have a flat, dendritic-like appearance, if grown under optimal conditions (Figure 1).

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 $1x10^6$ **viable** cells/mL in Starvation Medium.

(A)



(B)

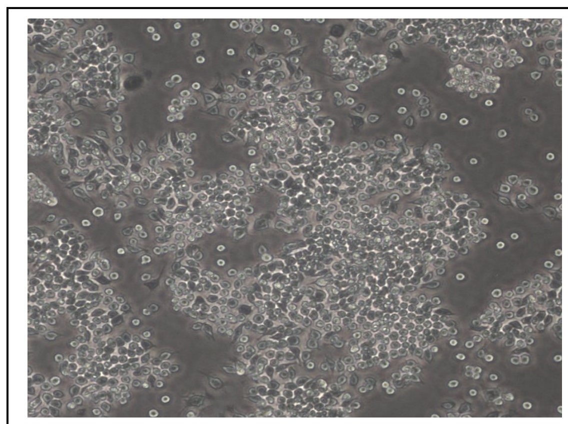


Figure 1. Morphology of RAW264.7 cells. (A) Morphology of cells suitable for the chemotaxis assay. (B) Morphology of cells which should not be used for the chemotaxis assay.

7. Fibronectin Coating

See images in Appendix 13.4

7.1 Dilute 25 µL of stock (1 mg/mL) fibronectin in 1X PBS to a final volume of 5 mL.

7.2 Add 30 µL to the electrode on the underside of each well; be careful not to pierce the electrode.

7.3 Incubate at room temperature for 30 minutes, aspirate, and allow to air dry for 30 minutes.

7.4 Repeat steps 7.2 and 7.3 with the upper side of the wells.

7.5 Coated plates may be stored under sterile conditions for up to 2 weeks at 4°C.

8. Experimental Procedure

The procedure described below is based on reference 2.

8.1 Add 160 µL of the test reagents to their respective wells in the bottom chamber of the CIM-Plate 16 (a meniscus should be formed at the top of each well; see figures in Appendix 13.3).

8.2 Attach the upper chamber of the CIM-Plate 16 carefully to avoid creating bubbles.

8.3 Add 25 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 cell suspension to the top wells (1 x 10^5 cells/well).

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

8.8 Place chamber in the instrument and start the protocol (see instrument settings in Appendix 13.6).

8.9 Acquire data for 72 hours.

9. Calculations

9.1 Calculate area under the curve (AUC) for the control and test samples using the 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. Acceptance Criteria

10.1 %CV for each control and test sample should be less than 30%.

11. 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

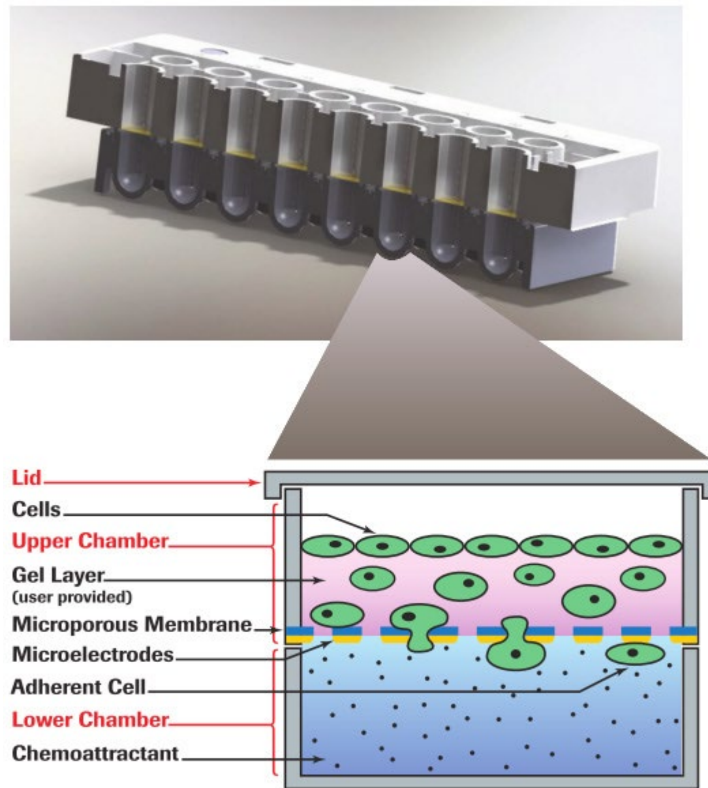
12. Abbreviations

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

13. Appendix

Images shown in sections 13.1-13.4 are reproduced from reference 2 with permission.

13.1 CIM-Plate 16 Schematic



13.2 ACEA CIM Plate 16 images

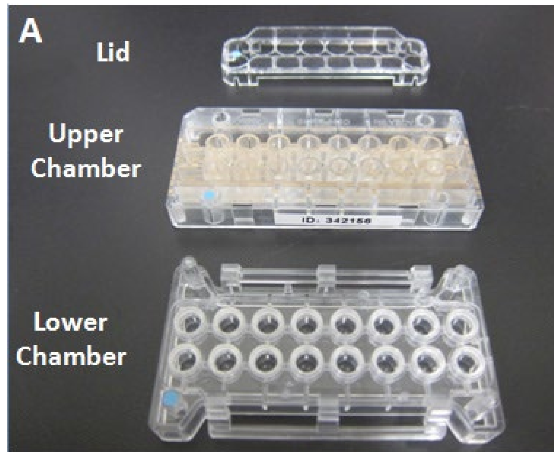


Fig A: ACEA CIM-Plate 16, unassembled.

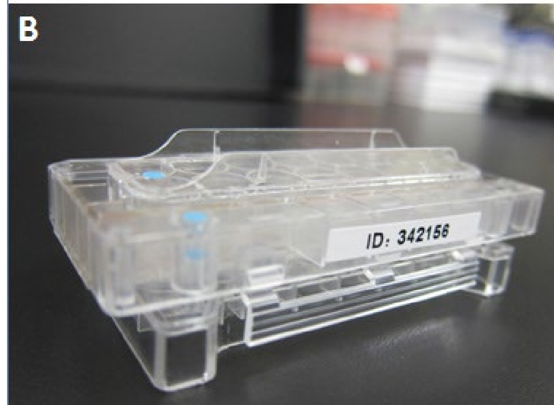
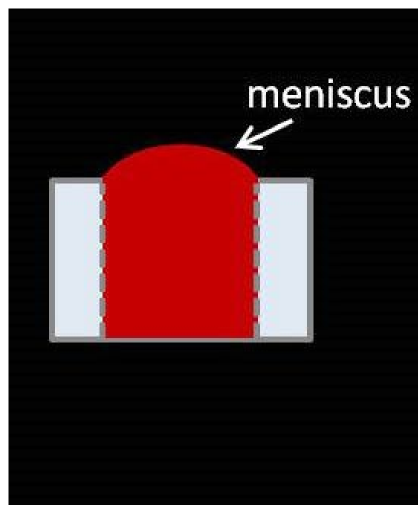


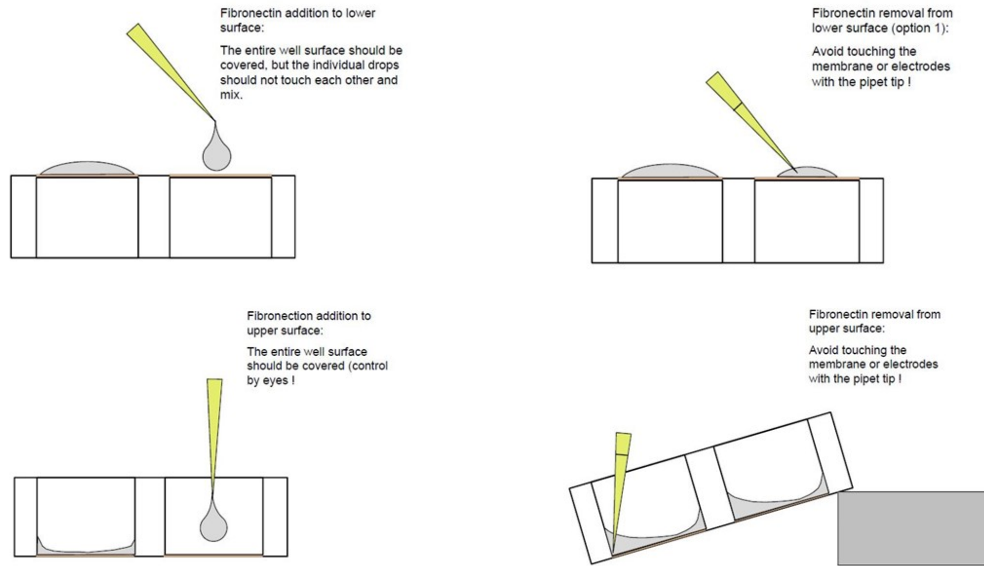
Fig B: ACEA CIM-Plate 16, assembled.

13.3 Reagent loading image



Sample loading procedure.
Plate bottom.

13.4 Fibronectin-coating images



13.5 Example of Assay Plate Map

	Plate 1		**Plate 2**		**Plate 3**	
	1A	**1B**	**2A**	**2B**	**3A**	**3B**
1	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated
2	PC	PC	PC	PC	PC	PC
3	Test 1	Test 1	Test 1	Test 1	Test 1	Test 1
4	Test 2	Test 2	Test 2	Test 2	Test 2	Test 2
5	Test 3	Test 3	Test 3	Test 3	Test 3	Test 3
6	Test 4	Test 4	Test 4	Test 4	Test 4	Test 4
7	Medium + Test 1	Medium + Test 1	Medium + Test 1	Medium + Test 1	Medium + Test 1	Medium + Test 1
8	Medium Only	Medium Only	Medium Only	Medium Only	Medium Only	Medium Only

13.6 Instrument settings

Step #	Step Name	Interval (min)	Sweeps
1	Background	1.00	1
2	Cell Seed	1.00	1
3	Cell Attach	0.50	500
4	Cell Chemotaxis	15.0	300