



## **NCL Method ITA-40**

### **Understanding the Role of Scavenger Receptor A1 in Nanoparticle Uptake by Murine Macrophages**

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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

Phagocytic cells take up nanoparticles via multiple routes, including phagocytosis and pinocytosis [1]. Nanoparticles with an anionic surface or made of anionic polymers are often phagocytosed via scavenger receptor A1 (SR-A1); multiple uptake routes may also be involved in the uptake of the same particle [2]. Understanding the route of nanoparticle uptake helps provide mechanistic insight into biological responses to and biodistribution of nanoparticles. The protocol described herein utilizes the murine macrophage cell line RAW 264.7 and its genetically engineered clone ½. The engineered cells were transduced with a lentivirus encoding SR-A1 specific siRNA; as the result, these cells do not express SR-A1 [2]. A decrease in the nanoparticle uptake by the SR-A1 deficient cells compared to the parent cells indicates the involvement of the SR-A1 pathway, as was verified using various models and inhibitors [2]. This protocol is intended for mechanistic studies.

## 2. Principles

This document describes a protocol for assessing the uptake of Cy-5 labeled nanoparticles by parent RAW cells, expressing SR-A1 receptor, and clone ½, deficient in SR-A1 expression, using a multispot reader. If the only goal of the experiment is to monitor the uptake by parent RAW cells, Alexa488 or FITC labeled particles can be used. Clone ½ cells have inherent low level green fluorescence due to the GFP-tagged lentiviral construct incorporated into the genome of these cells [2]; therefore, this green autofluorescence interferes with the accurate assessment of the uptake of nanoparticles tagged with a green fluorophore. The AID reader described in this protocol is equipped with filters for monitoring FITC and Cy5 fluorophores. Other methods, such as flow cytometry, can be used to monitor particle uptake. In this case, the fluorophores attached to the particles should match the specifications of the flow cytometer used in the experiment. The uptake of unlabeled metallic nanoparticles by parent RAW cells and clone ½ can also be tracked using elemental analysis such as ICP-MS.

When an AID multispot reader is used to monitor the uptake, the analysis results in two readouts – number of spots and activity. The number of spots reflects the number of cells that took up nanoparticles; this measure is equivalent to the percent positive cells readout in flow cytometry. The activity readout reflects the brightness of the fluorescent signal and, therefore, indicates the efficiency of uptake by a single cell; this measure is equivalent to the mean

fluorescent intensity readout in flow cytometry. Experimental steps involving instrument settings, plate reading and analysis are based on a series of technical documents by Autoimmun Diagnostica Gmb [3-6].

### 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 RPMI-1640 (Cytiva, HyClone, SH30096.01)
- 3.1.2 RPMI-1640, Phenol Red Free (Cytiva, SH30605.01)
- 3.1.3 Penicillin streptomycin solution (Cytiva, Hyclone, SV30010)
- 3.1.4 L-glutamine (Cytiva, Hyclone, SH30034.01)
- 3.1.5  $\beta$ -mercaptoethanol (Gibco, 21985-023)
- 3.1.6 Fetal bovine serum (FBS) (Cytiva, Hyclone, SH30070.03)
- 3.1.7 Phosphate buffered saline (PBS) (Cytiva, SH30256.01)
- 3.1.8 Trypan Blue solution (Gibco, 15250-061)
- 3.1.8 Distilled or sterile water

#### 3.2 Materials

- 3.2.1 Pipettes covering the range of 0.05 to 10 mL
- 3.2.2 Polypropylene tubes, 50 and 15 mL
- 3.2.3 Reagent reservoirs
- 3.2.4 75 cm<sup>2</sup> cell culture flasks
- 3.2.5 96-well plates (Optical bottom; Thermo Fisher, 165305) or commercial Elispot (various)

#### 3.3 Cell Lines

- 3.3.1 RAW 264.7 (ATCC, TIB-71)
- 3.3.2 RAW 264.7 SAR1 KO (Clone ½)

- 3.4 Equipment
  - 3.4.1 Centrifuge
  - 3.4.2 Refrigerator, 2-8°C
  - 3.4.3 Freezer, -20°C
  - 3.4.4 Cell culture incubator with 5% CO<sub>2</sub> and 95% humidity
  - 3.4.5 37°C water bath
  - 3.4.6 Biohazard safety cabinet approved for level II handling of biological material
  - 3.4.7 Inverted microscope
  - 3.4.8 Vortex
  - 3.4.9 Hemocytometer or automated cell counter

#### **4. Reagent and Control Preparation**

- 4.1 Complete RPMI-1640 medium
  - 10% FBS (heat inactivated)
  - 2 mM L-glutamine
  - 100 U/mL penicillin
  - 100 ug/mL streptomycin sulfate
  - 50 µM β-mercaptoethanol

Store at 2-8°C protected from light for no longer than 1 month. Before use, warm in a water bath.
- 4.2 Heat-inactivated fetal bovine serum

Thaw a bottle of FBS at room temperature, or overnight at 2-8°C and allow to equilibrate to room temperature. Incubate 30 minutes at 56°C in a water bath, mixing every 5 minutes. Fifty (50) mL single use aliquots may be stored at 2-8°C for up to one month or at a nominal temperature of -20°C indefinitely.
- 4.3 Negative Control

Use PBS as a negative control. Process it the same way as your study samples.
- 4.4 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 1 mL of Cy5-labeled nanoparticles. Each nanoparticle sample is plated 4 times. Concentration or a series of concentrations of nanoparticles is selected for each nanoparticle experimentally. The highest non-cytotoxic concentration with fluorescence sufficient for the AID reader to detect is recommended for this protocol. Parent RAW cells and control nanoparticles, e.g. Cy-5 labeled, drug-free liposomes with a total lipid concentration of ~0.5-1 mg/mL, can be used for such preliminary studies.

## 6. Cell Preparation

Grow cells in complete culture media, using conditions specific for the individual the cell lines. On the day of the experiment, count the cells; if cell viability is  $\geq 80\%$ , concentrate the cells in complete culture media to a concentration of  $5.0 \times 10^4$  cells/mL.

*Cells concentration may need to be adjusted accordingly for different treatment times.*

## 7. Experimental Procedure

### 7.1 Assay Plate Preparation and Treatment:

- 7.1.1 Add 100  $\mu$ L of cells (either RAW 264.7 or the SRA1 KO clone) from step 6 to the appropriate wells in a 96-well plate (5000 cells/well).  
*See Appendix in Section 11.*
- 7.1.2 Allow the cells to attach at room temperature for 20 - 30 minutes, then incubate overnight at 37°C and 95% CO<sub>2</sub>.
- 7.1.3 Prepare the samples at 2X of the final concentrations defined in the research plan.
- 7.1.4 Seed 100  $\mu$ L of prepared samples to the appropriate wells.  
(It is advisable to use positive samples in wells A1, A12 and H12 to facilitate stage calibration.)

7.1.5 Incubate for 24 hours at 37°C and 95% CO<sub>2</sub>.

*Note: incubation time may differ depending on assay conditions.*

7.1.6 After incubation, wash the cells twice with Phenol Red-free medium. Add and aspirate the medium gently to avoid loss of cells.

7.1.7 Add 50 µL of Phenol Red-free medium and read the plate using the EliSpot reader.

*Alternately, after washes, treat with 4% PFA for 15 minutes at room temperature, aspirate the PFA, quickly rinse with water and blot on a paper towel. Cover with foil and keep at 4°C until ready to read.*

## 7.2 AID Reader Start-up and Plate Read:

***Refer to the instrument and software manuals for more detailed instructions.***

7.2.1 Turn on the AID multispot reader, light sources and computer.

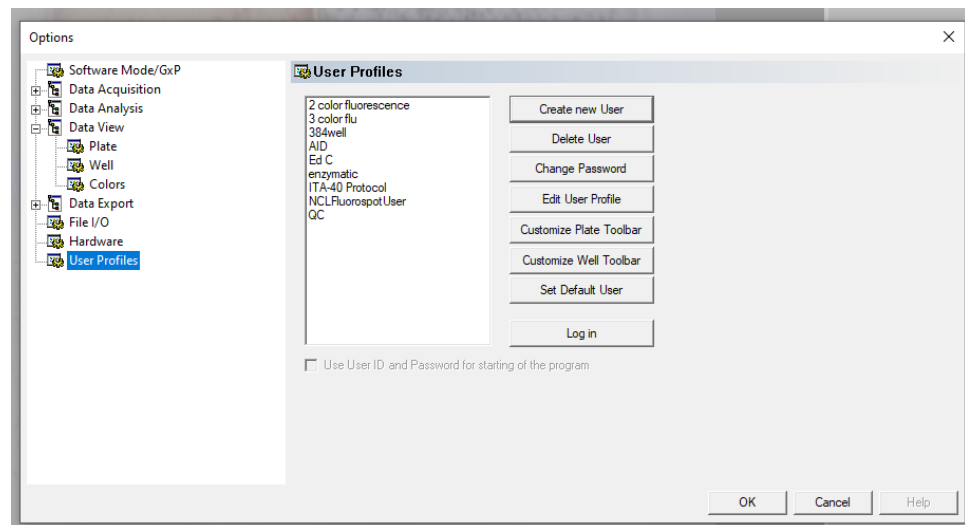
7.2.2 Launch the AID software and choose the **EliSpot** button.



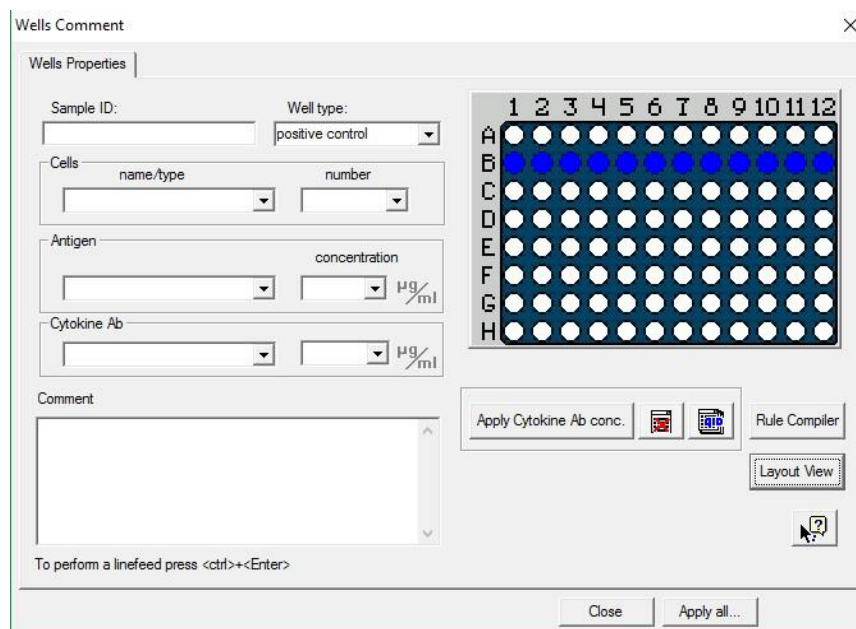
7.2.3 Above the menu bar check the user profile. If it reads **User: ITA-40 Protocol** go to step 7.2.6. If not, under **File** on the menu, click on **Options**, then click **User Profiles** in the newly opened Options window.



- 7.2.4 In the user profile window, highlight **ITA-40 Protocol**, click **Log In**, enter the password (*lab specific*) then click **OK**.
- 7.2.5 Click **OK** in the Options window to close.

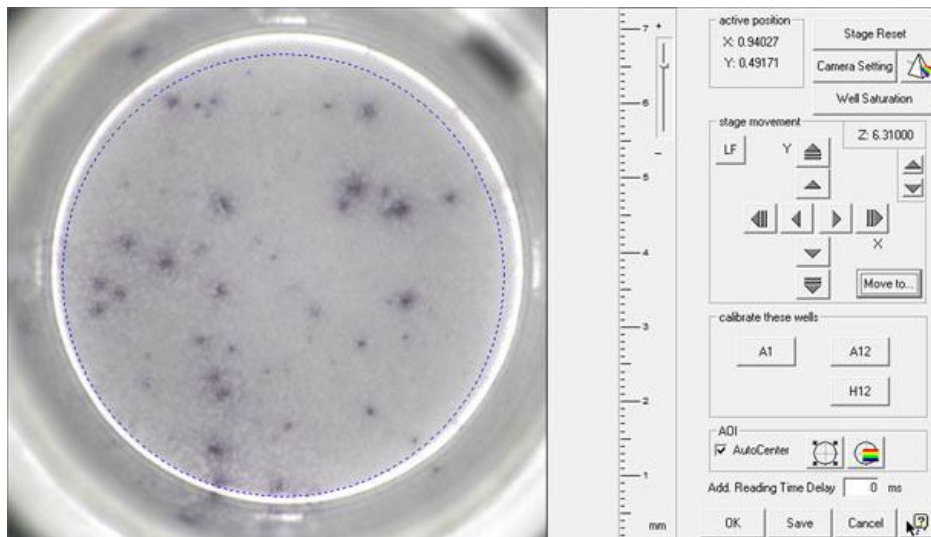


- 7.2.6 Under the **File** menu click **New**. This should open a plate template with the ITA-40 settings. Remember to save the file under a new name.
- 7.2.7 To add information on cell lines used, cells/well and treatments click on the **Tools** menu, click **Plate Layout**, then click **Edit Layout**. This will open the **Wells Properties** window.





- 7.2.8 Under the **Tools** menu, choose **Calibrate Stage** and choose well **A1**; center and adjust imaging ring to encompass most of the well. Do the same for wells **A12** and **H12**. Click **Save** and **OK** to save the stage calibration.

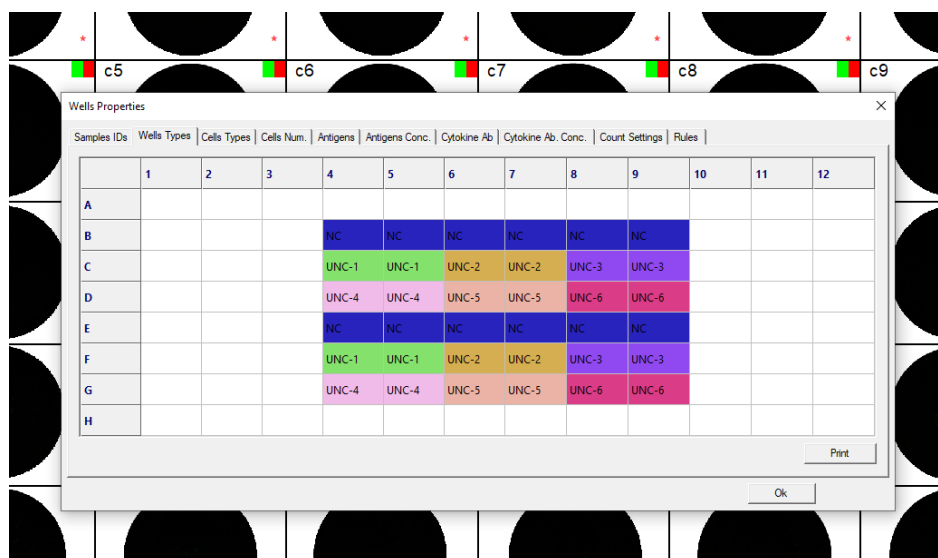


- 7.2.9 On the plate map, use the mouse to select the wells to be read then right click and highlight **Read, Count & Analyze** on the drop-down menu.
- 7.2.10 After the plate has been read, click the **Save** button.

## 8. Results and Analysis

*If necessary, the analysis algorithms and count settings may be changed from the AID default. This may be done before or after the plate read.*

- 8.1 Once the plate has been read and analyzed, click the **Excel** button on the menu bar. This will open an Excel file with the spot counts and activities for each of the selected wells.
- 8.2 Images of the plate and individual wells may be selected and captured using the **Power Point** button on the menu bar or by using the Print Screen function.
- 8.3 Plate and well information may be captured by clicking **Tools, Plate Layout**, then **View Layout**.



## 9. References

1. Liu Y, Hardie J, Zhang X, Rotello VM. Effects of engineered nanoparticles on the innate immune system. *Semin Immunol.* 2017 Dec;34:25-32.
2. França A, Aggarwal P, Barsov EV, Kozlov SV, Dobrovolskaia MA, González-Fernández Á. Macrophage scavenger receptor A mediates the uptake of gold colloids by macrophages in vitro. *Nanomedicine (Lond).* 2011 Sep;6(7):1175-88.
3. Introduction to the AID *cytoSpot* Reader (AID Autoimmun Diagnostica GmbH 2025)
4. User Guide AID *EliSpot* 8.0 (AID GmbH 2021)
5. Quick Guide for the AID *EliSpot* Software 7.0 (AID Autoimmun Diagnostica GmbH 2021)
6. Introduction to the AID *multiSpot* (AID Autoimmun Diagnostica GmbH 2021)

## 10. Abbreviations

AID	Advanced Imaging Devices (Autoimmun Diagnostika)
FBS	fetal bovine serum
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
RT	room temperature

## 11. Appendix

### Example Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC											PC
B												
C		Untreated	Untreated	T1	T1	T2	T2	T3	T3	T4	T4	
D		Untreated	Untreated	T1	T1	T2	T2	T3	T3	T4	T4	
E		Untreated	Untreated	T1	T1	T2	T2	T3	T3	T4	T4	
F		Untreated	Untreated	T1	T1	T2	T2	T3	T3	T4	T4	
G												
H												PC

**Wells A1, A12, H12:** RAW 264.7 cells + PC for stage calibration

**Rows C, D:** RAW 264.7 cells + Cy5-NP

**Rows E, F:** RAW 264.7 SRA1 KO (Clone ½) + Cy5-NP