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Amnis® NFκB Translocation Kit User's Guide

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Application

Nuclear Factor kappa B (NFκB) transcription factor plays a central role in regulating many key processes in mammalian cells, including differentiation, cell growth, inflammation, immunity, and stress responses. The p50/p65 heterodimer is considered to be the most abundant form of NFκB. NFκB is held in an inactive state in the cytoplasm by its inhibitor IkB. Phosphorylation of serine residues on the IκB proteins marks them for degradation via ubiquitination, therefore allowing activation of the NFκB complex. Activated NFκB complex translocates into the nucleus. The Anti-Hu NFκB Alexa Fluor® 488 antibody recognizes NFκB p50 which is a component of the NFκB p50/p65 heterodimer.[1,2]

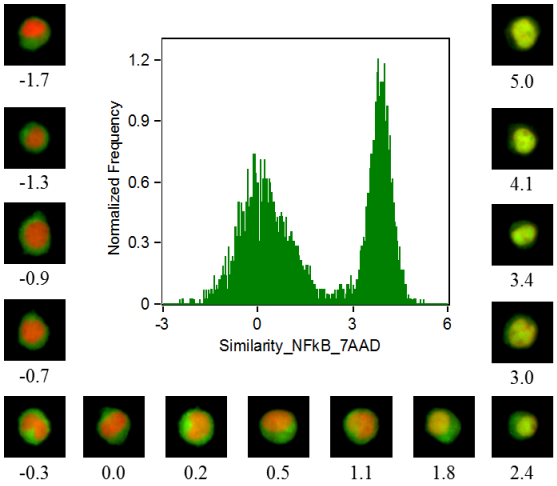
The activation of NFκB and its translocation to the nucleus has become a focal point for studying cellular function, signal transduction pathways, disease mechanisms and drug discovery efforts. Classical biochemical techniques are semi-quantitative in nature and do not provide per-cell translocation measurements. Manual microscopy allows visual identification of nuclear translocation on a per-cell basis but an objective and statistically rigorous assessment is difficult to obtain. The Amnis® FlowSight® & ImageStream®^{X} platforms overcome these problems by combining the quantitative power of flow cytometry with the spatial information provided by microscopy in one system. This kit along with the Amnis imaging cytometry platforms measures NFκB translocation in an objective, reproducible and statistically robust manner.

Test Principle

This is a unique assay on Amnis® FlowSight® & ImageStream®^{X} platforms that provides translocation information not easily obtainable by traditional methods. The two-color assay kit rapidly detects and quantitates NFκB translocation from the cytoplasm to the nucleus.

The Nuclear Localization Wizard in the Amnis IDEAS® software creates an analysis template for measuring the nuclear localization of a probe, in this case NFκB. This wizard uses the Similarity feature which calculates a pixel by pixel correlation of the nuclear 7-AAD image to the anti-Hu NFκB Alexa Fluor® 488 image. A high Similarity score indicates the two images are highly correlated and similar, whereas low scores indicate the images are dissimilar. In Figure 1 a histogram and 60x composite images of NFκB and 7-AAD is shown that demonstrates varying Similarity scores between the anti-Hu NFκB Alexa Fluor® 488 (green) image and the 7-AAD nuclear image (red) in untreated and LPS treated THP-1 cells.

Figure 1: Histogram and 60x composite images of NFκB and 7-AAD demonstrating varying Similarity scores between the anti-Hu NFκB Alexa Fluor® 488 (green) image and the 7-AAD nuclear image (red) of untreated and 1 µg/mL LPS treated THP-1 cells.



Workflow

This protocol was developed using 10^6 cells per sample.

Prepare stimulated and control cells, including positive, negative and single color controls

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Centrifuge cells and wash with PBS, then transfer cells with 1 x 10^6 cells per test into each tube

↓

Prepare working solutions of 1X Fixation buffer (20 µL 5X Fixation solution and 80 µL 1X PBS per test), 0.25X Fixation buffer (5 µL 5X Fixation solution and 95µL 1X PBS per test) and 1X Assay Buffer (0.4 mL 5X assay buffer and 1.6 mL dH_2O per test)

↓

Optional - Stain with surface markers (follow the surface markers protocols)

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Optional - Wash with PBS or 1X Assay Buffer to remove any excess surface marker probe and pellet cells

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Fix the cells with 1X Fixation Buffer for 10 min at room temperature

↓

Wash and pellet with 1X Assay Buffer

↓

Prepare working solution of Anti-Hu NFκB Alexa Fluor® 488 Antibody/Permeabilization Buffer by adding 5 µL anti-Hu NFκB antibody to 95 µL permeabilization buffer per test

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Add 100 µL of the Anti-Hu NFκB Alexa Fluor® 488 Antibody/Permeabilization Buffer to each test, mix thoroughly and incubate for 30 min at room temperature

↓

Wash and pellet with 1X assay buffer

↓

Resuspend cells in 100 µL 0.25X Fixation buffer

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Add 10 µL 7-AAD per test incubate 5 min

↓

Run samples (including single color controls) on the ImageStream®^{X} or FlowSight®

↓

Analyze data files in IDEAS® using the Nuclear Localization wizard

Kit Components

- Anti-Hu NFκB(p50) Alexa Fluor® 488 (Part Number 4700-1674, 50 tests/vial)
  - Clone: 4D1
  - Isotype: Mouse IgG1, κ
  - Reactivity: Human
- 7-AAD Reagent (Part Number 4000-0290, 50 tests/vial)
- 5X Fixation Solution (Part Number 4300-0340, 2–3 mL vials)
- 5X Assay Buffer (Part Number CS202124, 50 mL/vial)
- Permeabilization Buffer (Part Number CS202125, 10 mL/vial)

Materials Not Supplied

- ImageStream®^{X} or FlowSight® imaging cytometer
- SpeedBead® ImageStream® System Calibration Reagent (Catalog No. 400041) or FlowSight® Calibration Beads (Catalog No. 400300)
- 10% Bleach
- Coulter Clenz® (Beckman Coulter , Item No. 8546931)
- 70% Isopropanol
- Cells of interest
- Stimulation reagent
- Tissue culture instruments and supplies
- Siliconized polypropylene (Low Retention) Microcentrifuge tubes 1.5 mL (Fisherbrand Part No.02-681-320 or equivalent)
- Micropipettors
- Disposable micropipettor tips
- Tabletop centrifuge capable of exceeding x300G
- Vortex mixer
- 1X Phosphate buffered saline (PBS) without calcium or magnesium
- Deionized water
- Optional: Round-bottom 96-well plate (Becton Dickinson, Catalog No. 353077) and plate cover (X-Pierce™ film, Catalog No. Z722502, Sigma-Aldrich)

Precautions

- Wear proper laboratory attire (lab coat, disposable nitrile gloves, safety glasses) when handling or using this product.
- Anti-Hu NFκB Alexa Fluor® 488 antibody and 7-AAD solution, 5X Assay Buffer, and Permeabilization Buffer contain sodium azide, which is toxic if ingested. Reagents containing sodium azide should be considered a poison. If products containing sodium azide are swallowed, seek medical advice immediately and show product container or label. (Refer to NIOSH, National Institute for Occupational Safety and Health; CAS#: 2628-22-8; and also to GHS, the Globally Harmonized System of Classification and Labeling of Chemicals.) Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic gas. Any reagents containing sodium azide should be evaluated for proper disposal. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent build-up in plumbing. Check with regulatory agencies to determine at what concentration sodium azide may cause a product to be regulated as hazardous.
- Fixation solution contains Formalin. Please refer to the Safety Data Sheet (SDS) for specific information on hazardous materials.
- All fluorochrome conjugated antibodies are light sensitive and must be stored in the dark at 2 to 8°C
- Avoid microbial contamination of the solution, which may cause erroneous results.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- During storage and shipment, small volumes of product may become entrapped in the seal of the product vial. For maximum recovery of the product, centrifuge the vial briefly prior to removing the cap.
- Do not use the reagent beyond the expiration date.
- Safety Data Sheets (SDSs) for kit reagents are available from our website (www.luminexcorp.com) or by contacting Luminex Technical Support.

Storage

Upon receipt, store the kit as follows:

2-8°C:

- Anti-Hu NFκB Alexa Fluor® 488 antibody
- 7-AAD Reagent
- 5X Assay Buffer
- Permeabilization Buffer

Room temperature:

- 5X Fixation Solution

CAUTION: Fluorochrome-conjugated antibodies should always be stored at 4°C. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.

Before You Begin

This protocol was developed as an assay on the Amnis® FlowSight® and ImageStream®^{X} platforms to measure nuclear translocation of NFκB. For optimal staining, cell concentrations should be 1 x 10^{6} cell/test. Care should be taken to keep cell concentrations as constant as possible in all samples of an experiment.

Cells should be acquired shortly after the sample preparation has been completed. While cells are fixed, and some cell lines have been shown to yield stable results for up to 24 hours, post preparation stability may be different for your particular cell line.

The following procedures for cell staining are guidelines. Different cell types may have more optimal permeablization and staining conditions. You may need to adjust the amount of reagents used for optimal staining of your cell samples.

Time considerations: The process of staining cells with the Amnis NFκB translocation kit takes approximately 1 hour and 10 min. Acquiring data on an ImageStream^{X} or FlowSight can vary depending on your cell concentration and number of samples and how many events are collected. Analysis time will vary depending on the number of events acquired and how many files you are analyzing.

Preparation of Buffers

Prepare all buffers fresh each day of your experiment. To ensure there is enough buffer for your samples mix n+2 samples, where n is the number of tests you wish to run for each experiment, and one for each single color control.

1. 1X fixation buffer working solution. Mix the following reagents together.

Component	1 Test	25 Tests	50 Tests
5X Fixation Solution	20 µL	500 µL	1 mL
1X PBS	80 µL	2000 µL	4 mL
Total Volume	100 µL	2500 µL	5 mL

2. 0.25 fixation buffer working solution. Mix the following reagents together.

Component	1 Test	25 Tests	50 Tests
5X Fixation Solution	5 µL	125 µL	250 µL
1X PBS	95 µL	2375 µL	4750 µL
Total Volume	100 µL	2500 µL	5000 µL

3. 1X Assay Buffer working solution. Mix the following reagents together.

Component	1 Test	25 Tests	50 Tests
5X Assay Buffer	0.4 mL	10 mL	20 mL

Component	1 Test	25 Tests	50 Tests
dH_2O	1.6 mL	40 mL	80 mL
Total Volume	2 mL	50 mL	100 mL

4. Anti-Hu NFκB Alex Fluor® 488 Antibody/Permeabilization Buffer working solution. Mix the following reagents together.

Component	1 Test	25 Tests	50 Tests
Anti-Hu NFκB Alex Fluor® 488	5 µL	125 µL	250 µL
Permeabilization Buffer	95 µL	2375 µL	4750 µL
Total Volume	100 µL	2500 µL	5000 µL

Example Cell Staining Protocol

Staining is done in siliconized polypropylene (NOT POLYSTYRENE) microcentrifuge tubes to prevent cell loss. All washes are done at 300xg for 5 min at 4°C in a swinging bucket rotor.

Each test is 1 x 10^6 cells.

1. Prepare required buffers as described under "Preparation of Buffers" on page 5.
2. Prepare samples for testing. Each Test is for 1 x 10^6 cells. Regardless of type of cells or culture vessel used, each experiment should include the proper negative, positive and single color control samples as indicated below.
   - **Negative control samples:** The negative control should be a sample from your cell culture, not treated to induce NFκB translocation.
   - **Positive control sample:** The positive control should be a sample from your cell culture, that has been treated to have full NFκB translocation. Here are 2 examples for positive control samples, in each case culture cells should be in mid-exponential growth:
     – HL-60 cells 10 ng/mL of tumor necrosis factor (TNFα) for 30 min should result in full NFκB translocation.
     – THP-1 cells 1 µg/mL Lipopolysaccharide (LPS) for 1 hour should result in full NFκB translocation.
   - **Single-color control samples:** Label a sample with only the anti-Hu NFκB Alexa Fluor® 488. Label a sample with only 7-AAD. For both cases it does not matter if the sample is stimulated or not for NFκB translocation. These sample are used to make the compensation matrix.
3. After treating cells to induce translocation of NFκB wash cells with PBS and centrifuge cells at 300xg for 5 min.
4. **Optional**: Aspirate off the supernatant and stain with surface markers (follow the surface markers protocols). Wash with PBS or 1X Assay buffer and centrifuge cells at 300xg for 5 min to remove any excess surface markers.
5. Aspirate off the supernatant and fix the cells with 100 µL 1X Fixation buffer, mix thoroughly and incubate for 10 min at room temperature.

   NOTE: Do not over fix the cells, this might result in poor labeling results.

6. Wash with 1 mL 1X assay buffer and centrifuge cells at 300xg for 5 min.
7. Aspirate off the supernatant. Add 100 µL of anti-Hu NFκB Alexa Fluor® 488/Permeabilization Buffer working solution to each test, mix thoroughly and incubate for 30 min at room temperature.
8. Wash with 1 mL 1X assay buffer and centrifuge cells at 300xg for 5 min.
9. Aspirate off the supernatant and resuspend cells in 100 µL 0.25X fixation buffer.

   NOTE: If you would like higher cell concentration per µL you can add between 50–100 µL 0.25X fixation buffer per sample.

10. Add 10 µL 7-AAD per test incubate 5 min.
11. Keep samples in 1.5-mL siliconized polypropylene tubes. If your instrument has an auto sampler you can transfer your samples to a round bottom 96 well plate (Becton Dickinson, Catalog No. 353077) with plate cover (X-Pierce™ film, Catalog No. Z722502, Sigma-Aldrich).
12. Samples are now ready for acquisition on the ImageStream®^{X} or FlowSight®.
13. Run samples within 24 hr.

    NOTE: If you are not running the sample right away store them in the dark at 2-8°C.

Data Acquisition

1. Acquire your samples and single color controls on the Amnis® ImageStream®^{X} or FlowSight® instruments.
2. Detailed instructions on using the systems are available in the *INSPIRE™ User'sManual* for the Amnis ImageStream^X or FlowSight instruments.
3. Use the following INSPIRE version:
   - ImageStream^X – 4.1.501 or later
   - ImageStream^X Mk II – 200.0.755 or later
   - FlowSight – 100.2.256 or later
4. NFκB and 7-AAD are both excited by the 488-nm laser. Use a sample with all fluorochromes in your experiment to set the laser powers so that there is no saturation on the camera. NFκB is in Channel 2 and 7-AAD will be in Channel 5.

   NOTE: 7-AAD is also excited by the 561-nm laser but will still be in Channel 5. Use of the 561-nm laser is optional.

5. Acquire data files for each single color compensation control sample.

   NOTE: When collecting the single color controls it is critical that the side scatter laser (785-nm laser) and the brightfield are OFF and all channels are enabled.

   NOTE: Before acquiring the anti-Hu NFκB Alexa Fluor® 488 single-color control run 10% bleach then PBS like a sample to remove any leftover 7-AAD nuclear stain in the tubing.

6. Acquire experimental sample files.

Data Analysis

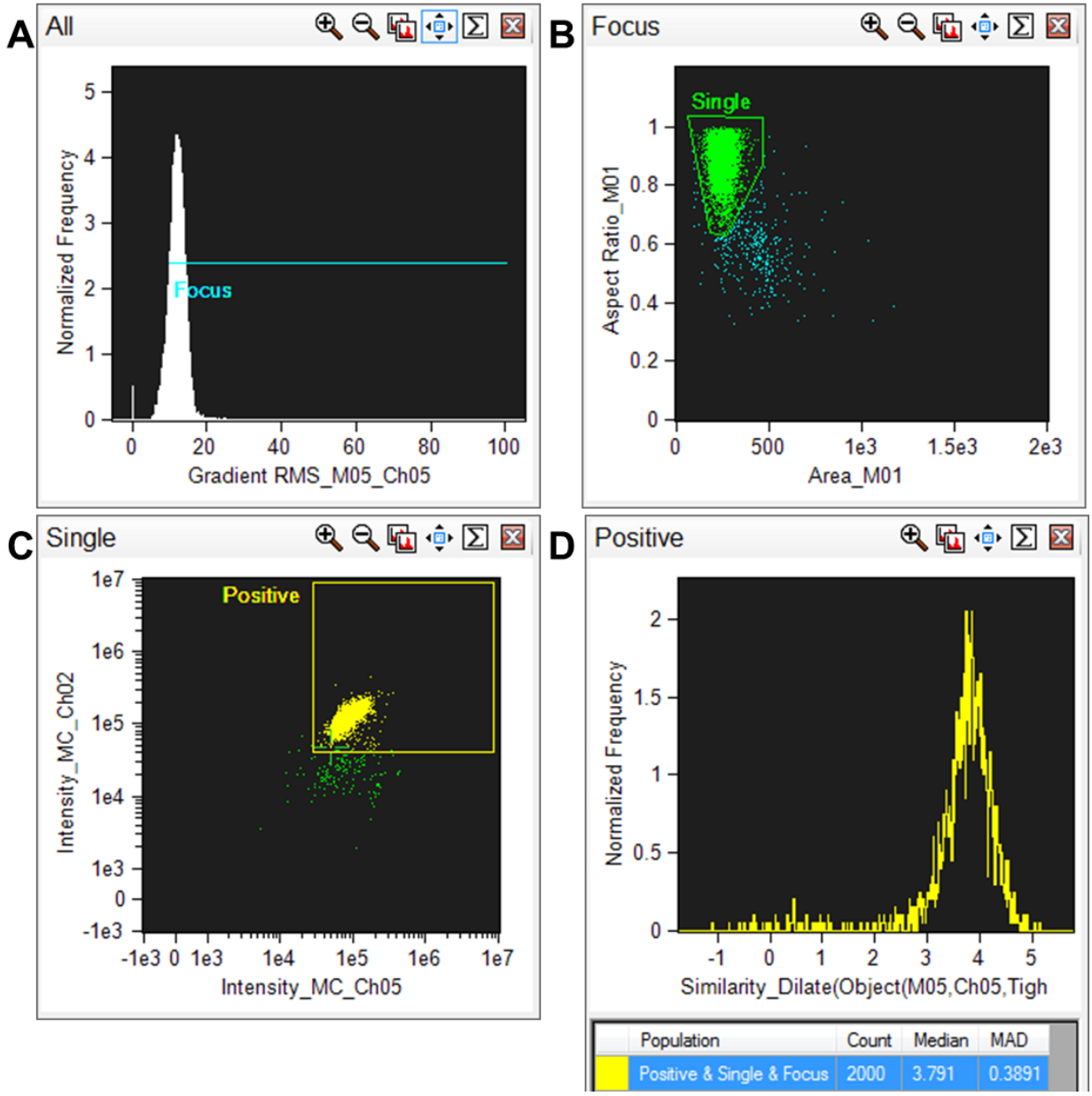
The sequence of steps in data analysis is as follows. For detailed instructions refer to the *IDEAS® User's Manual*.

Use IDEAS 6.0 or later.

- Open IDEAS and click on '**Start Analysis**'
- Select Data file to open, click '**Next**'
- Click '**New Matrix**' The compensation wizard opens. Or you may select a matrix previously created.
    - **Step 1: Select the control files for compensation.** Click 'Add files' and select your single color control files. Click 'Next'
    - **Step 2: Select/remove channels for compensation.** For this kit you will use Channels 2 and 5 and any additional channels you used for surface staining. Check appropriate boxes and click 'Next'
    - **Step 3: Validate the compensation matrix.** Click 'Finish' and save the .ctm file. The Best Fit method is recommended and the best fit lines can be validated by double-clicking on the coefficient values. For more details on validating compensation see the IDEAS User manual. The compensation wizard ends and the matrix is added. Click 'Next' You may add an Analysis template if you have one.
    - **Step 4: Name your file.** Click 'Next' It is recommended to keep the default names
    - **Step 5: Set image display properties.** Select the channels used in your experiment (for this kit make sure Channel 2 and 5 are selected). Brightfield and side scatter channels are automatically checked. Click 'Next'
    - **Step: 6 Select a wizard to begin analysis.** Double-click on **Nuclear Localization wizard** and follow the instructions in the wizard. If unsure of how to gate, read the tips in the wizard window.
- **Step 1: Select translocation image channels.** From the drop down menus select Ch05 (7-AAD) for the Nuclear Image and Ch02 (NFκB) for the Translocating Probe. Click 'Next'.
- **Step 2: Gate cells in best focus**. A Gradient RMS histogram of the All population has been added to the analysis area. Click on the bins in the histogram to view the images in each bin. The cells with better focus have higher Gradient RMS, values. Begin your region at the bin after the Gradient RMS value you wish to exclude and continue the region to the maximum in the plot as shown in Figure 2A. Click 'Next'.
- **Step 3: Gate single cells.** A scatter plot of Area versus Aspect Ratio has been added to the analysis area. Single cells will have an intermediate Area value and a high Aspect Ratio. Click on the dots to view the image associated with that dot. Note that the image is surrounded by a light green line and the image next to it in the image gallery is not it's neighbor in the plot. The images are presented in the order of acquisition. Draw a region that includes single cells as shown in Figure 2B. Click 'Next.'
- **Optional:** If there are surface stains in your experiment Click 'Yes' to the question that pops up 'Do you want to analyze sub-populations?' otherwise, Click 'No'.
- **Step 4: Gate double positives.** Draw a region that includes double-positive cells on the nuclear intensity (7-AAD Channel 05) vs. translocating probe intensity (NFκB Channel 02) scatter plot as shown in Figure 2C.
- **Step 5: Gate translocated events.** A histogram has been added of the Similarity feature of the last gated population of cells that are positive for both 7-AAD and NFκB as shown in Figure 2D. If there is evidence that a subpopulation of cells has high Similarity scores draw a region that includes cells with high translocation. If the distribution is Gaussian and the whole population shifts with high Similarity scores for the positive control and low for the negative control it may be more appropriate to report the Fischer's discriminant ratio (RD) based on the median and mad of the population. See Cytometry A. 2011 Jun;79(6):461-9. doi: 10.1002/cyto.a.21068. Click 'Finish' and save analysis data file.

  The similarity score represents how similar the NFκB image is to the 7-AAD image. Low similarity scores indicate the images are not similar (NFκB is not translocated to the nucleus) where as high similarity scores indicate the images are similar (NFκB is translocated to the nucleus).

Figure 2: Sequence of Analysis Steps in Evaluating NFκB Translocation: A represents Step 2: Gate cells in best focus. B represents Step 3: Gate on single cells. C represents Step 4: Gate double positives. D represents Step 5: Gate translocated events and is the Similarity histogram that represents how similar the NFκB image is to the 7-AAD image.



Sample Results

Figure 3: Example ImageStream® Data. Histograms for the negative control (blue) and the 1 µg/mL LPS treated (red) THP-1 cells are shown, along with representative 60x images with median Similarity scores. The brightfield

image, anti-Hu NFκB Alexa Fluor® 488 (green), 7-AAD (red) and a composite of the anti-Hu NFκB Alexa Fluor® 488 and 7-AAD images are shown.

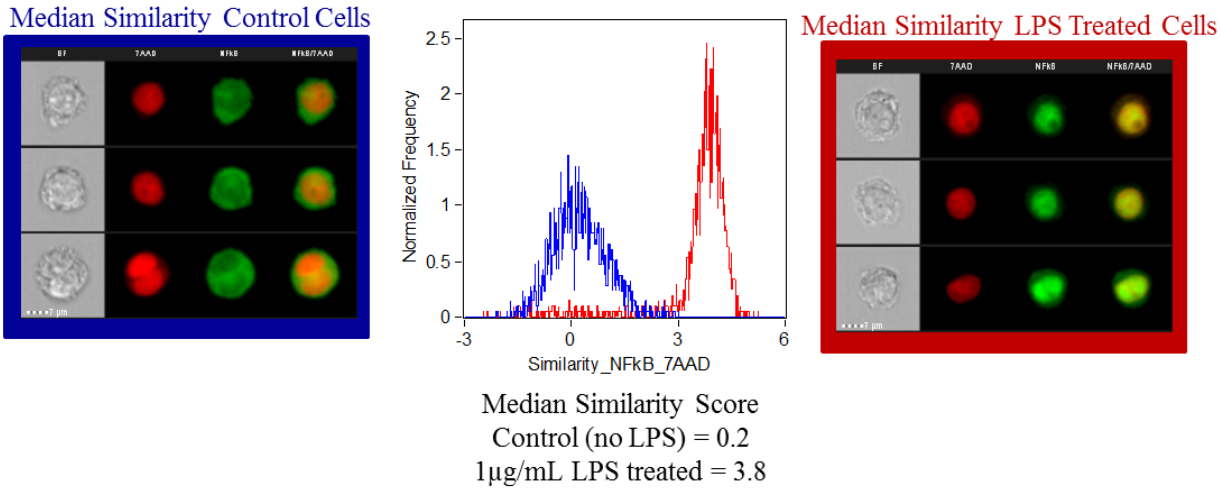


Figure 4: Example FlowSight® Data. Histograms for the negative control (blue) and the 10 ng/mL TNFα treated (red) HL-60 cells are shown, along with representative images with median Similarity scores. The brightfield image, anti-Hu NFκB Alexa Fluor® 488 (green), 7-AAD (red) and a composite of the anti-Hu NFκB Alexa Fluor® 488 and 7-AAD images are shown.

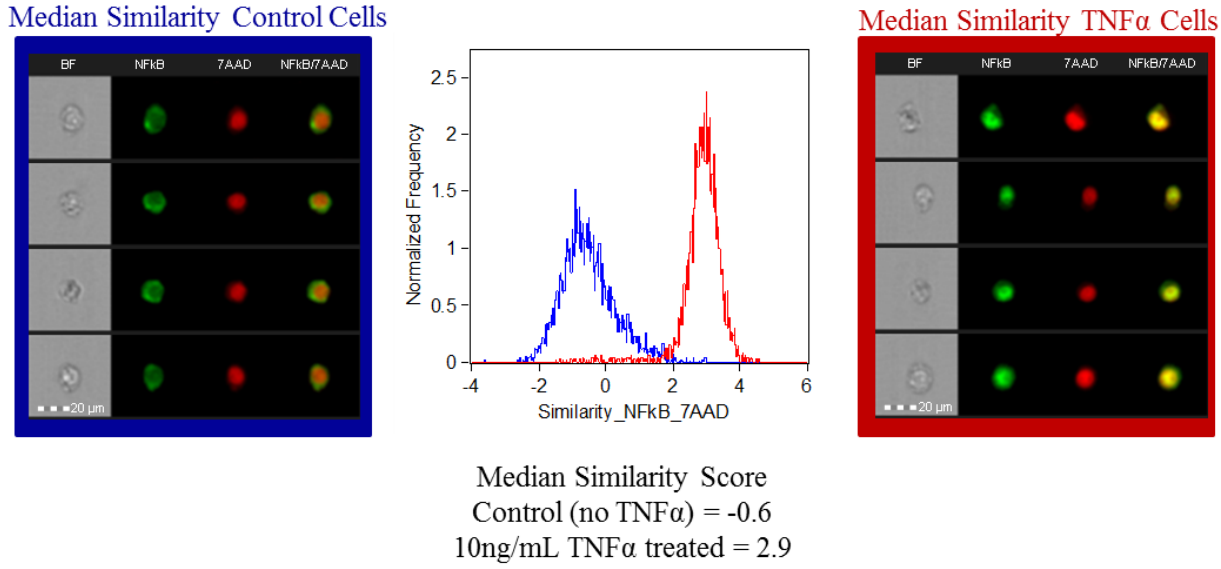
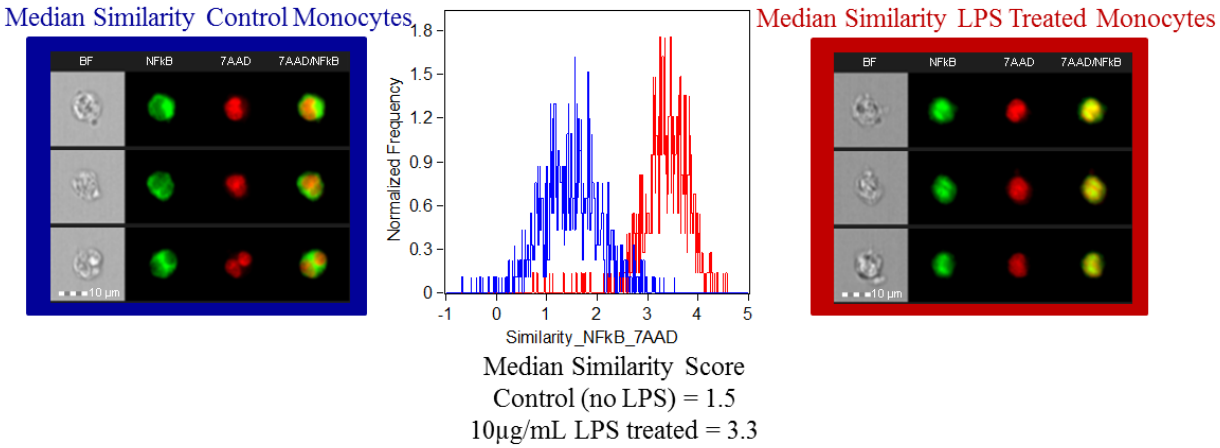


Figure 5: Example ImageStream Data. In this study peripheral blood was stimulated with 10 µg/mL LPS. LPS stimulation of monocytes activates several intracellular signaling pathways including the NFκB pathway. Human monocytes are identified by CD14. Histograms for the negative control (blue) and 10 µg/mL LPS treated (red) for the monocytes are shown. The CD14+ monocytes have a clear shift in their Similarity score from the control to the LPS treated blood. Representative 40x images of the brightfield image, anti-Hu NFκB Alexa Fluor® 488 (green), 7-

AAD (red) and a composite of the anti-Hu NFκB Alexa Fluor® 488 and 7-AAD images are shown with median Similarity scores.



Median Similarity Score
Control (no LPS) = 1.5
10µg/mL LPS treated = 3.3

Technical Hints

All kit reagents, should be brought to room temperature and mixed on a mechanical vortex prior to staining.

- Confirm that cells have greater than 80% viability prior to treatment for NFκB activation.
- Treatments for NFκB translocation must be optimized at the researcher's own discretion.
- To ensure optimal staining, avoid mixing of multiple kit lots.

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically; instrument clogging; too many cells	• In the event that you experience significant sample clumping and instrument clogging filter the sample through 80-µm mesh before loading onto the ImageStream®^{X} or FlowSight®. • If the sample is too concentrated you can dilute the sample with 1X PBS or 0.25X fixation buffer.
Too few cells	• Spin down cells and pool samples if necessary. • Make sure you are using siliconized polypropylene low-retention microcentrifuge tubes. Permeabilized cells stick to polystyrene which may result in too few cells.

Potential Problem	Experimental Suggestions
Background staining and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure. • Washing the samples with Permeabilization Buffer after labeling my also decrease background staining.
Dim anti-Hu NFκB Alexa Fluor® 488 Staining	• Dim staining obtained with the antibody may indicate reagent degradation. Verify that the reagent is not past its expiration dates before using. • Dim staining may also be a sign that the cell concentration was too high (>1 x 10^6 cells/test) and thus the concentration of reagents was insufficient to properly stain the cells. Repeat the experiment, using a lower number of cells per well. • Dim staining may be a sign of over fixation. If the cells are fixed for too long (more than 10 min with 1X fixation buffer) it may become difficult to permeabilize the cell, resulting in poor staining. • Dim staining may also be a sign that the incubation time was not sufficient to properly stain the cells. Repeat the experiment with longer incubation time.
Dim 7-AAD staining	• To have good uniform 7-AAD staining the samples need to be permeabilized with the permeabilization buffer prior to adding the 7-AAD. **Note:** For 7-AAD single color control follow the same labeling steps as the samples being labeled with anti-Hu NFκB Alexa Fluor® 488 but add only permeabilization buffer (not anti-Hu NFκB Alexa Fluor® 488/Permeabilization Buffer working solution). • Dim staining may also be a sign that the cell concentration was too high (>1 x 10^6 cells/test). If this is the case more 7-AAD can be added to the sample tube.
Single color anti-Hu NFκB Alexa Fluor® 488 antibody control has 7-AAD in it	Before acquiring the anti-Hu NFκB Alexa Fluor® 488 single color control run 10% bleach then PBS as if they were a sample to remove any leftover 7-AAD nuclear stain in the sample line tubing.

References

1. Maguire O, Collins C, O'Loughlin K, Miecznikowski J, Minderman H. Quantifying nuclear p65 as a parameter for NF-κB activation: Correlation between ImageStream cytometry, microscopy, and Western blot. *Cytometry* Part A. 2011;79(6):461-469.
2. Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene*. 2006;25(51):6680-6684.

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