Detection of neutral lipids in single-cell organisms using the Attune NxT Flow Cytometer

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Introduction

Eukaryotic cells such as adipocytes, organisms such as protozoa and algae, and some prokaryotes are able to store neutral lipids in lipid bodies [1]. Neutral lipid storage is of particular interest not only because such lipids can be used for fuel [2], but also because they are associated with pathogenesis: mycobacteria use host lipids to store neutral lipids when exposed to environmental stress [3,4]. Importantly, *Mycobacterium tuberculosis* loaded with neutral lipids can be detected in sputum of tuberculosis patients, indicating a relevant role for such storage during infection [5].

Microscopy or thin-layer chromatography (TLC) are often used for detection of neutral lipid storage; however, these methods are time-consuming and do not allow for high-throughput screening. Flow cytometry provides fast quantification of neutral lipid storage at the single-cell level, allows for direct medium- to high-throughput analysis, and potentially enables correlation with other parameters such as markers for cell viability and/or stress [6].

Here we demonstrate how to detect neutral lipids in the model organism *Mycobacterium marinum* using the Invitrogen™ Attune™ NxT Flow Cytometer, which is ideally suited for a variety of cell types as it allows for

gentle and safe analysis without clogging the instrument or wasting cells. The Attune NxT Flow Cytometer was developed with acoustics-assisted hydrodynamic focusing technology, with advanced fluidics designed to minimize clogging and effectively handle a broad range of cell types. This allows for a higher degree of data, detail, and throughput that enables processing of a large range of sample types—including large clumpy cells and samples with a low concentration of cells—quickly and accurately with no loss in data quality. In this application note we describe the staining of neutral lipids with the lipophilic Invitrogen™ BODIPY™ 493/503 dye, which is detected with the 488 nm excitation laser and 530/30 nm emission filter (BL1 detector).

Materials

- Middlebrook 7H10 agar plate supplemented with OADC (oleic acid, albumin, dextrose, catalase), cultured with *Mycobacterium marinum* strain M
- Middlebrook 7H9 medium supplemented with ADC (albumin, dextrose, catalase) and 0.05% Tween™ 80 (Merck Millipore), referred to as 7H9
- PBS supplemented with 0.1% Tween 80 (Merck Millipore), referred to as PBS Tween 80
- BODIPY 493/503 dissolved in ethanol to 1 mg/mL (Thermo Fisher Scientific, Cat. No. **D3922**)
- 4% paraformaldehyde (Thermo Fisher Scientific, Cat. No. **R37814**)
- Attune NxT Flow Cytometer with Blue/Red/Violet/Yellow Lasers (Thermo Fisher Scientific, Cat. No. **A24858**)
- Spectrophotometer and disposable cuvettes
- Microcentrifuge tubes (1.5 mL)
- Erlenmeyer flasks
- 50 µm mesh filter

Methods

Incubation

1. Inoculate a single colony of *M. marinum* strain M into 7H9 medium and grow to mid-log phase at 30ºC under shaking conditions (120 rpm).
2. Measure OD_{600} of bacterial culture.
3. Harvest 3 OD units and centrifuge for 5 minutes at 16,000 x *g*.
4. Remove supernatant and suspend in 10 mL PBS Tween 80.
5. Incubate for 2 days at 30ºC while shaking (120 rpm).
6. Inoculate a fresh culture of *M. marinum* in 7H9 medium.

Neutral lipid staining

1. Measure OD_{600} of bacterial cultures.
2. Collect 5 x 10^7 bacteria per sample.
3. Centrifuge sample at 16,000 x *g* and remove the supernatant.
4. Suspend pellet in 100 µL of 4% paraformaldehyde and incubate for 30 minutes at room temperature.
5. Add 800 µL of PBS and centrifuge for 5 minutes at 16,000 x *g*.
6. Prepare 10 µg/mL of BODIPY 493/503 staining solution in PBS.
7. Remove the supernatant and suspend the pellet in 100 µL of the BODIPY 493/503 staining solution.
8. Incubate for 30 minutes on ice, protected from light.
9. Add 800 µL of PBS and centrifuge for 5 minutes at 16,000 x *g*.
10. Remove the supernatant and suspend the pellet in 200 µL of PBS.
11. Filter the sample into a microcentrifuge tube using a 50 µm mesh filter; keep sample on ice and protected from light.

Sample acquisition

1. Turn on the computer and the cytometer; run the start-up and performance tests.
2. Create a new experiment.
3. Name the BL1 channel “BODIPY 493/503”.
4. Insert a logarithmic SSC-A vs. FSC-A density plot.
5. Insert a logarithmic FSC-A vs. FSC-H density plot.
6. Insert a logarithmic histogram for BODIPY 493/503.
7. Acquire the positive control sample without recording, and adjust the FSC threshold empirically until the bacterial population is visible. Adjust the SSC, FSC, and BODIPY 493/503 voltages to improve visualization. The following settings are good starting voltages for *M. marinum*: SSC voltage at 360 mV, FSC voltage at 360 mV and BODIPY 493/503 voltage at 450 mV.
8. Gate the most dense population in the SSC-A/FSC-A density dot plot, followed by an FSC-A/FSC-H gate for single-cell identification (Figure 1).

9. Start the acquisition with the following criteria:

   a. Volume = 100 µL

   b. Flow rate = 12.5 µL/min

   c. Record collection and stop the acquisition when 20,000 events in the singlet gate is reached.

   d. In case the bacterial concentration is not optimal, adjust the acquisition criteria to improve appropriate uptake of the sample. Please note that the sample should be <1,000 events/second when measuring at 12.5 µL/min to avoid coincidence.

Data analysis

M. marinum forms aggregates, which can be observed by the typical smear in the FSC-A/SSC-A plots of *M. marinum* cultured in standard 7H9 medium (Figure 1A, 1B). Less mycobacterial aggregation is observed in the PBS Tween 80 positive control, visualized by an increase of events with lower FSC/SSC values (Figure 1C). In order to compare samples within experiments, any cell coincidence or aggregation effect needs to be avoided and excluded; therefore, an FSC/SSC gate is used on the small and dense population present in all samples. Subsequent FSC-H/FSC-A gating was performed to gate on singlet cells (Figure 1D–1F).

Results and discussion

BODIPY 493/503 is a lipophilic dye that stains neutral lipids present in bacteria. This dye heavily accumulates in neutral lipid–rich compartments such as lipid bodies. Unstained 7H9-cultured *M. marinum* and unstained PBS Tween 80–incubated *M. marinum* do not show fluorescence (Figure 2A), but upon BODIPY 493/503 staining, a slight increase is observed for 7H9-cultured *M. marinum* (Figure 2B). This indicates the presence of neutral lipids in *M. marinum*, though this slight increase is not indicative of the presence of neutral lipid–rich compartments. Indeed, neutral lipids can be stored in compartments or localize in the cell wall [7-9]. Previous work has shown that mycobacteria cultured in 7H9 do not contain lipid bodies [5]. Unlike the nutrient-rich 7H9 condition, incubation in PBS with Tween 80 leads to a strong increase in BODIPY 493/503 fluorescence (Figure 2B), indicative of storage of neutral lipids in compartments, which was validated by microscopy (data not shown). Previous work showed that *M. marinum* can grow on Tween 80 as a single carbon source in minimal medium [10], but when growth is restricted due to limitation of nutrients as observed here in PBS Tween 80, neutral lipids are stored instead (Figure 2B).

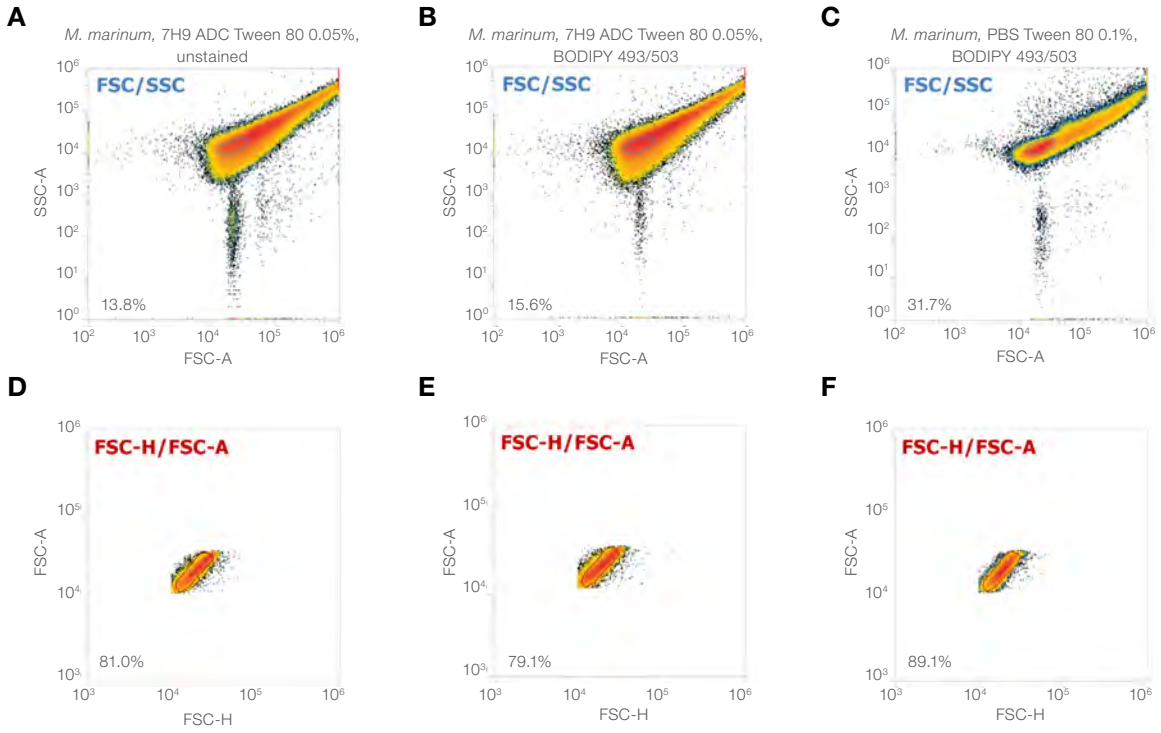

Figure 1. Gating strategy. Unstained and BODIPY 493/503–stained *M. marinum* samples were first gated on FSC-A/SSC-A **(A–C)** to prevent aggregation effects on analysis, followed by a singlet gate on FSC-H/FSC-A **(D–F)**. Gate percentages are indicated in the lower left corner of each plot.

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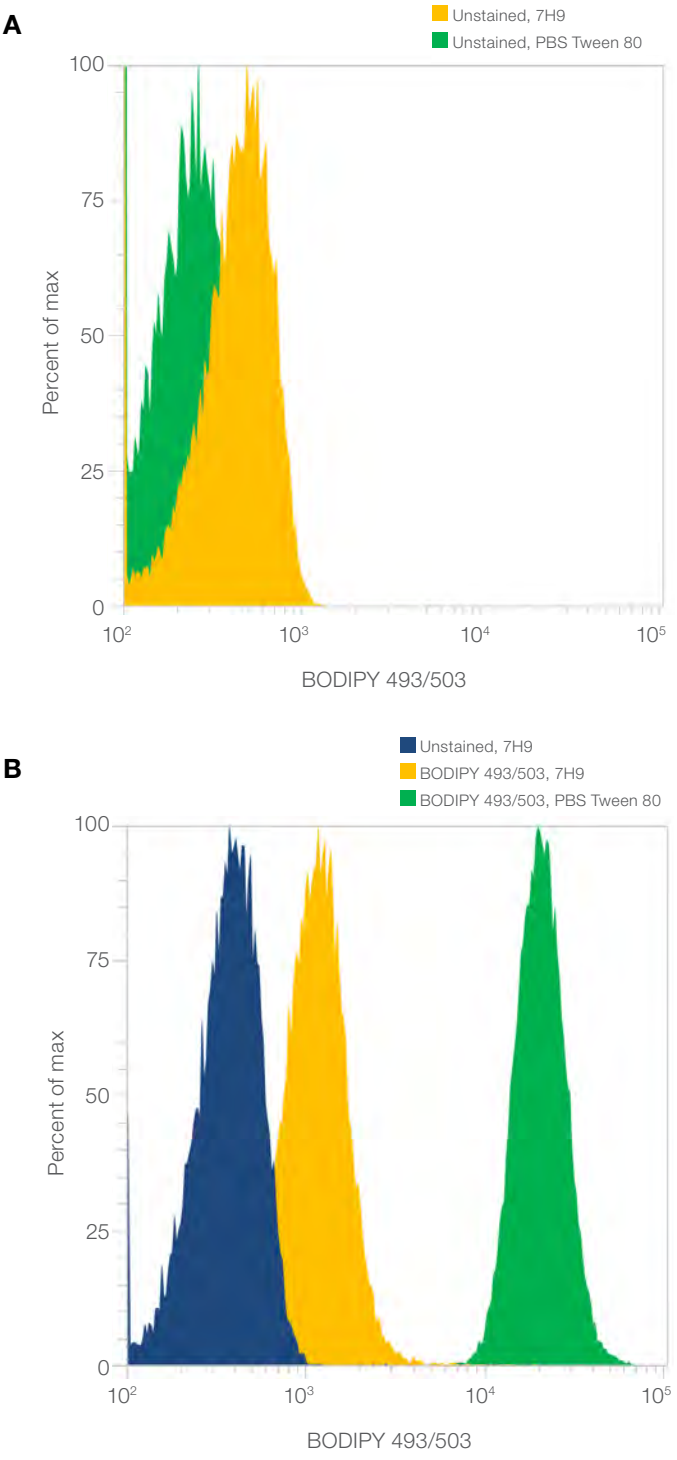

Figure 2. BODIPY 493/503 staining. Overlay histograms of unstained *M. marinum* **(A)** and BODIPY 493/503–stained *M. marinum* **(B)** samples in 7H9 medium and PBS Tween 80, given as percentage of max.

Conclusion

Storage of neutral lipids by mycobacteria can be evaluated on an Attune NxT Flow Cytometer using BODIPY 493/503. This will not only help further understanding of the role and function of mycobacterial neutral lipid storage in pathogenesis, but also enable use as a tool to extrapolate neutral lipid staining to other prokaryotes and eukaryotes. To verify storage of neutral lipids in lipid bodies, microscopic examination is recommended.

References

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