Better, Stronger, Faster - your BY-onic Cytometer

August 15, 2018
Dolores Ciufio, FAS
<table>
<thead>
<tr>
<th>Instrument</th>
<th>Lasers (excitation beam); Pre-set configuration (nm)</th>
<th>Number of fluorescent detectors (by laser)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I Laser</td>
<td>II Laser</td>
</tr>
<tr>
<td>Cytek Aurora</td>
<td>488</td>
<td>405</td>
</tr>
<tr>
<td>BD LSR II-1</td>
<td>488</td>
<td>405</td>
</tr>
<tr>
<td>BD LSR II-2</td>
<td>488</td>
<td>405</td>
</tr>
<tr>
<td>BD LSR-Fortessa</td>
<td>488</td>
<td>405</td>
</tr>
<tr>
<td>ThermoFisher Attune NxT</td>
<td>488</td>
<td>561</td>
</tr>
</tbody>
</table>
Agenda

Attune® NxT™ Systems
  Instrument
  Autosampler

Fluidics and Optics

Best practices

Software overview
Traditional hydrodynamic focusing

Particle positioning in laser is important

- **Focused laser**
  - Hydrodynamic core
  - Low sample flow rate (e.g., 12 µL/min)

- **Focused laser**
  - High sample flow rate (e.g., 200 µL/min)

**Narrow particle focus = Narrow distribution**

**Broad particle focus = Broad distribution**

- **Count** vs. **Intensity**

Thermo Fisher Scientific
Acoustic assisted hydrodynamic focusing

High sample input flow rates allow for more sample flexibility

12.5 µL/min

1,000 µL/min

Narrow particle focus = Narrow distribution

Prior to wrapping in sheath
Acoustic focusing

End-on view of capillary

Before

After
Acoustic Focusing Capillary

Acoustic Waves – similar to ultrasound used to visualize a fetus in utero

~20cm

Piezo-electric device

Focused Particles/Cells

Capillary

200 um

Flow

Laser
10µm high
50µm width

• Flow rate can be increased while maintaining resolution
Clog resistant

Acoustic focusing

200 um diameter flow cell / ≥ 200 um fluid lines

Sample syringe delivery
Attune® NxT System

Status indicator lights
Green, Amber, Blue, Multicolor

Any tube that fits on the tube lifter will work
On board fluids

1ml Sample Syringe

Color coded Fluid Lines

Black Sensor Connections

Color coded Fluid Lines

Volumetric delivery: Accurate counts Clog resistance

Notifies when filling/emptying is needed

Waste

Focusing Fluid

Wash Fluid

Shutdown Fluid
Beam Profile: Flat-Top Lasers

Flat-top vs. Gaussian Lasers
Flat top lasers reduce need for alignment adjustments

- consistent laser energy excitation
- ensures resulting data is due to biological, not instrument variation
## Blue Yellow - Configuration

<table>
<thead>
<tr>
<th>Blue</th>
<th>488 nm laser</th>
<th>50 mW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow</td>
<td>561 nm laser</td>
<td>50 nm</td>
</tr>
</tbody>
</table>

9 parameters (7 fluorescent detectors)

<table>
<thead>
<tr>
<th>Excitation Laser</th>
<th>Emission Filter (nm)</th>
<th>Channel</th>
<th>Recommended Dyes</th>
<th>Fluorescent Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue - 488 nm</td>
<td>530/30</td>
<td>BL1</td>
<td>Alexa Fluor® 488 FITC</td>
<td>eGFP Emerald eYFP</td>
</tr>
<tr>
<td></td>
<td>590/40</td>
<td>BL2</td>
<td>PE-Alexa Fluor® 610 PE-Texas Red® PE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>695/40</td>
<td>BL3</td>
<td>PE-Alexa Fluor® 700 Tri-Color® PE-Cy®5.5 PerCP PerCP-Cy®5.5 Qdot® 705</td>
<td></td>
</tr>
<tr>
<td>Yellow - 561 nm</td>
<td>585/16</td>
<td>YL1</td>
<td>PE</td>
<td>mOrange RFP eTomato</td>
</tr>
<tr>
<td></td>
<td>620/15</td>
<td>YL2</td>
<td>PE-Alexa Fluor® 610 PE-Texas Red®</td>
<td>mCherry DoRed mKate mStrawberry</td>
</tr>
<tr>
<td></td>
<td>695/40</td>
<td>YL3</td>
<td>PE-Alexa Fluor® 700 PE-Cy®5.5 Qdot® 705 Tri-Color®</td>
<td></td>
</tr>
<tr>
<td></td>
<td>780/60</td>
<td>YL4</td>
<td>PE-Cy®7 Qdot® 800</td>
<td></td>
</tr>
</tbody>
</table>

![Instrument Configuration Diagram](image-url)
Fluorescent protein optimization filters

Default filters

- **mCherry**
  - 615/25 BP filter
- **GFP**
  - 510/10 BP filter
  - 540/30 BP filter
- **YFP**
  - 525 dichroic LP

Instrument Configuration diagrams show filter combinations for mCherry, GFP, and YFP.
## Fluorescent proteins

Table 1. Spectral characteristics of fluorescent proteins commonly used in flow cytometry.

<table>
<thead>
<tr>
<th>Fluorescent protein</th>
<th>Excitation max (nm)</th>
<th>Emission max (nm)</th>
<th>Channel on Attune Nxt Flow Cytometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azurite, TagBFP, mTagBFP, mTagBFP2, Cerulean, ECFP, TagCFP, AmCyan</td>
<td>383, 400, 400, 400, 433, 439, 458, 458</td>
<td>450, 456, 456, 456, 456, 475, 476, 480, 489</td>
<td>VL1 (440/50), VL2 (512/25)</td>
</tr>
<tr>
<td>T-Sapphire</td>
<td>399</td>
<td>511</td>
<td>VL3 (603/48)</td>
</tr>
<tr>
<td>LSS-mKate1, LSS-mKate2</td>
<td>463, 460</td>
<td>624, 605</td>
<td>BL1 (530/30 or 510/10*)</td>
</tr>
<tr>
<td>TurboGFP, EGFP, TagGFP, emerald GFP (emGFP)</td>
<td>482, 483, 484, 487</td>
<td>502, 506, 507, 509</td>
<td>BL1 (530/30 or 510/10*)</td>
</tr>
<tr>
<td>TagYFP, TurboYFP, EYFP, Topaz, Venus, Citrine</td>
<td>508, 508, 514, 514, 515, 517</td>
<td>524, 524, 527, 527, 528, 529</td>
<td>BL2 (574/26 or 590/40)^ or (540/30)*</td>
</tr>
<tr>
<td>mKOM, mOrange, mOrange2, Kusabira Green Orange, E2 Orange</td>
<td>548, 548, 549, 548, 540</td>
<td>559, 562, 565, 561, 561</td>
<td>YL1 (585/16)</td>
</tr>
<tr>
<td>DsRed, DsRed2, DsRed-Express, tdTomato, TagRFP, mStrawberry, mCherry, mKate, mKate2, TurboFP635 (Katuhka)</td>
<td>553, 553, 553, 554, 555, 574, 587, 588, 588, 588</td>
<td>583, 583, 584, 581, 584, 596, 610, 635, 633, 635</td>
<td>YL2 (620/15 or 615/25*)</td>
</tr>
<tr>
<td>mPlum, HcRed, mRaspberry, mNeptune, E2Crimson</td>
<td>590, 592, 598, 599, 611</td>
<td>649, 645, 625, 649, 646</td>
<td>YL3 (695/40)</td>
</tr>
</tbody>
</table>

*Bandpass emission filter used with the Invitrogen™ Attune™ Nxt Fluorescent Protein Filter Kit (optional, Cat. No. 100022775). †The 574/26 filter set is standard on all Attune Nxt instruments that are not configured with a yellow laser. The 590/40 filter set is included on Attune Nxt instruments configured with the yellow laser.
Autosampler

- Plate formats: 96 or 384 well plates, standard or deep well
  **Round/U**, V-bottom and flat

- Plate experiments can include tubes
- Runs plate/records wells horizontally or vertically
- Mixes by pipetting up/down (user sets # of mixes)
- Probe is rinsed between wells (user sets # rinses)
Autosampler Mixing procedure

The user sets:
- The plate type
- The total sample volume
- The number of mixes
  (over mixing may cause bubbles)

The system defines:
- The liquid level in well
- The probe position
- The mixing method

Mixing sample by aspiration instead of shaking ensures homogeneity of the sample and maintains cell viability
Sample syringe / volumetric delivery

Paradigm shift

Sample is not automatically drawn from tube

Use the **Collection Panel Run Protocol** to control data acquisition

Sample is pulled from the tube when **Run** is clicked
Collection panel for plate experiment
Best Practice – dilute samples

Dilution is the solution!
Best practice: cell concentration and sample flow rate

The event rate will approach maximums stated in the column header when samples of stated concentrations are run at the flow rates below.

When acquiring large event files (i.e., files with > 10^6 events), plot parameters should not be changed while recording. Maximum file size: 20 Million events

<table>
<thead>
<tr>
<th>Sample flow rate</th>
<th>Maximum sample concentration (35,000 ev/sec)</th>
<th>Maximum sample concentration (8000 ev/sec) – for accurate counts</th>
<th>Flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 µL/ minute</td>
<td>2.1 x 10^6 cells/mL</td>
<td>0.48 x 10^6 cells/mL</td>
<td>- Particles &gt; 4 µm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Predominantly acoustic focusing</td>
</tr>
<tr>
<td>500 µL/ minute</td>
<td>4.2 x 10^6 cells/mL</td>
<td>0.96 x 10^6 cells/mL</td>
<td>- Particles &gt; 2 µm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Predominantly acoustic focusing</td>
</tr>
<tr>
<td>200 µL/ minute</td>
<td>6.7 x 10^6 cells/mL</td>
<td>1.5 x 10^6 cells/mL</td>
<td>- Smallest sample core</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Predominantly hydrodynamic focusing</td>
</tr>
<tr>
<td>100 µL/ minute</td>
<td>1.3 x 10^7 cells/mL</td>
<td>3 x 10^6 cells/mL</td>
<td>- Small particles &lt; 2 µm</td>
</tr>
<tr>
<td>25 µL/ minute</td>
<td>5.4 x 10^7 cells/mL</td>
<td>1.2 x 10^7 cells/mL</td>
<td>- Best resolution from background for dimly positives assays</td>
</tr>
<tr>
<td>12.5 µL/ minute</td>
<td>1.0 x 10^8 cells/mL</td>
<td>2.4 x 10^7 cells/mL</td>
<td>- Smallest sample core</td>
</tr>
</tbody>
</table>

Let your biology and data quality be your guide. If good data is obtained while running at 2-8,000 ev/sec, adjust the sample concentration and flow rate to maintain that.
Best practices – Use routines on the Instrument tab –

- **Recover Sample** - returns unused sample volume back to the well or the tube.
- **Rinse** - flushes system between samples. Runs automatically every time the SIP is lowered, but also can be user-initiated.
- **Sanitize Attune SIP** - sanitizes the SIP and sample lines between samples or experiments
- **Deep Clean** - thoroughly washes the system sample lines and flow cell between experiments
- **Debubble** - clears bubbles from the fluidics lines of the cytometer
- **Unclog** - back flush operation to remove clogs from the sample line.

Follow on screen instructions

All part of fluidics maintenance
Sample Recovery

Follow on-screen instructions

Anytime sample remains in the sample loop

Stop option has been reached
Operator clicks stop

NOTE: Must select recover sample before lowering the tube lifter
## Daily instrument cleaning guide

### Between samples
- **Rinse** – automatically initiated when SIP is lowered (for tubes), or set in *run protocol* for plates
- **Sanitize SIP** between sticky samples or cell counts

### Between users / experiments
- **Quick Deep Clean** - 30 minute cleaning routine (click on the arrow below the Deep Clean icon to select *Quick*)
- **2x Sanitize SIP / Sanitize Autosampler SIP** (plate experiments) –
  - 1\(^{st}\) with 10% bleach per instructions
  - 2\(^{nd}\) with MilliQ water.

### End of day
- **Thorough Shutdown** (click on the arrow below the Shutdown icon to select *Thorough*)

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**Note:** Always wipe the outside of the SIP after doing a SIP Sanitize
Summary: Better – Stronger - Faster

Better – acoustic focusing
  - volumetric delivery
  - automated cleaning routines
  - intuitive software

Stronger – clog resistant engineering
  - challenging samples
  - more stable laser alignment

Faster - 6 sample flow rates from 12.5 to 1000 µl/min
  - dilute samples
  - rare event analysis