Center for Advanced Research Technologies
Flow Cytometry Resource

UNIVERSITY of ROCHESTER MEDICAL CENTER

FCR Analyzer Training

Phase 1

The purpose of this training document is to familiarize prospective flow cytometer users with the process of running an experiment, highlighting critical steps that must be taken to ensure success and the capture of good, usable data. The procedure discussed herein is platform agnostic and intended to provide a high-level overview. Instrument-specific instruction will be available to users upon completion of this training module.

Basic Overview

1. Create a new experiment
2. Select detectors/Indicate fluorochromes
3. Establish sample structure and organization
4. Create single stain and unstained controls for compensation or unmixing
5. Adjust scatter and fluorescence detectors
6. Record compensation or unmixing controls
7. Calculate unmixing or compensation
8. Build a worksheet
9. Record samples
10. Save experiment and export and transfer all files
11. Clean the instrument

Experiment:

An essential component of operation in cytometer software. Experiments will contain and store all user-adjusted settings that are specific to the samples being run, as well as recorded sample files, worksheets, plots, and gating schemes created.

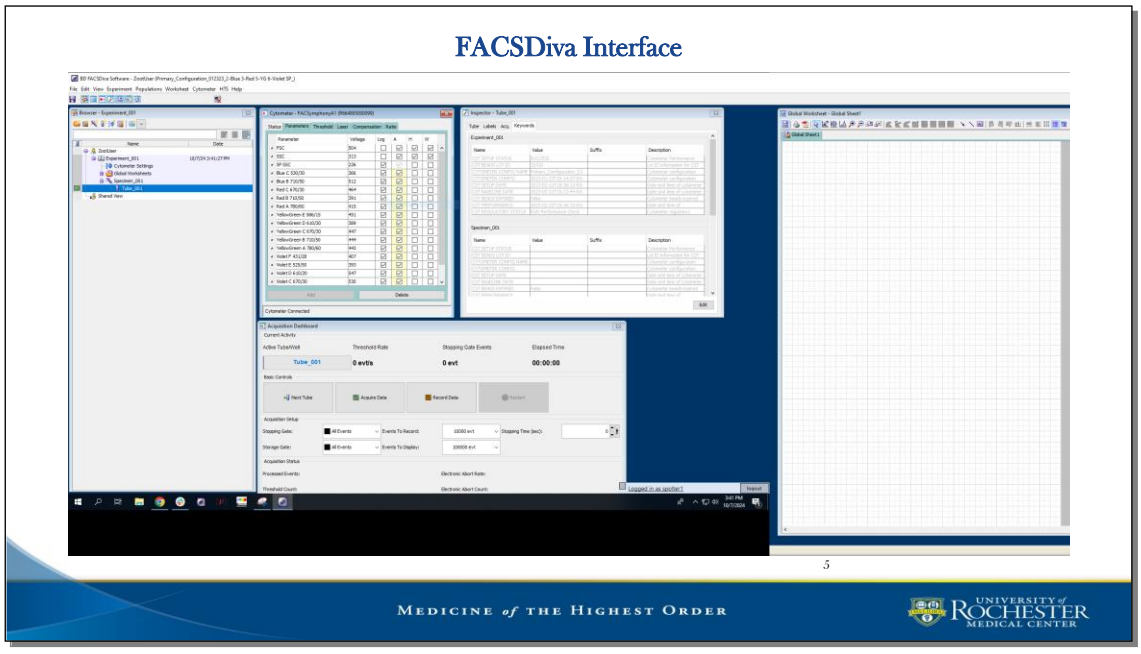
Terminology: It is important to be familiar with the terminology that you may encounter while using a conventional or full spectrum cytometer.

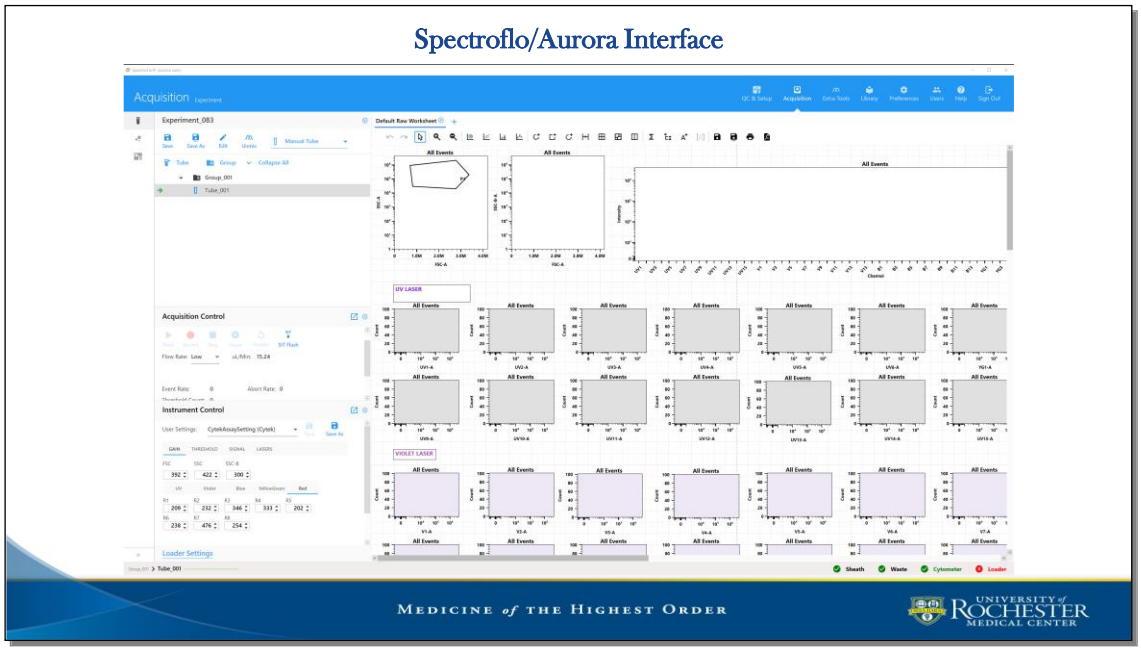
- Compensation/Unmixing
  - The mathematical process of visually correcting for fluorescent spillover/overlap from each fluorochrome in the panel into detectors where signal from another fluorochrome will be captured. (analogous to unmixing)
- Compensation control/Reference control
  - A single-stained control or unstained control used to calculate compensation/unmixing for fluorescent overlap
- Fluor/Fluorochrome
  - A fluorescent molecule/chemical used to label cells. Fluorochromes are often conjugated to antibodies. Fluorescent proteins are considered fluorochromes as well in the context of flow cytometry
- Voltage/Gain
  - Controls amplification of the signal being received by a detector
- Detector
  - The apparatus used to detect photons of emitted or scattered light. Signal can be amplified with voltage/gain
- Scatter (FSC and SSC)
  - Light scattered by particles that gives proportional information about size and complexity.
- Sample
  - Experimental samples and controls (FMO/treated vs untreated samples/etc) are considered samples in the experiment being created. These are separate from compensation/unmixing controls.
- SIP/SIT
  - Acronym: Sample Introduction Probe/Tubing (or Sample Injection Port/Tubing)

Getting Started

1. Log into Windows, if necessary, using the posted username and password.
2. Log into PPMS using your own username and password.
3. Check that the instrument is ready to use. The instrument should be powered on and connected to the software. Check sheath and waste tanks to make sure waste is not overfull and sheath is not low. Empty the waste and/or fill the sheath if needed.

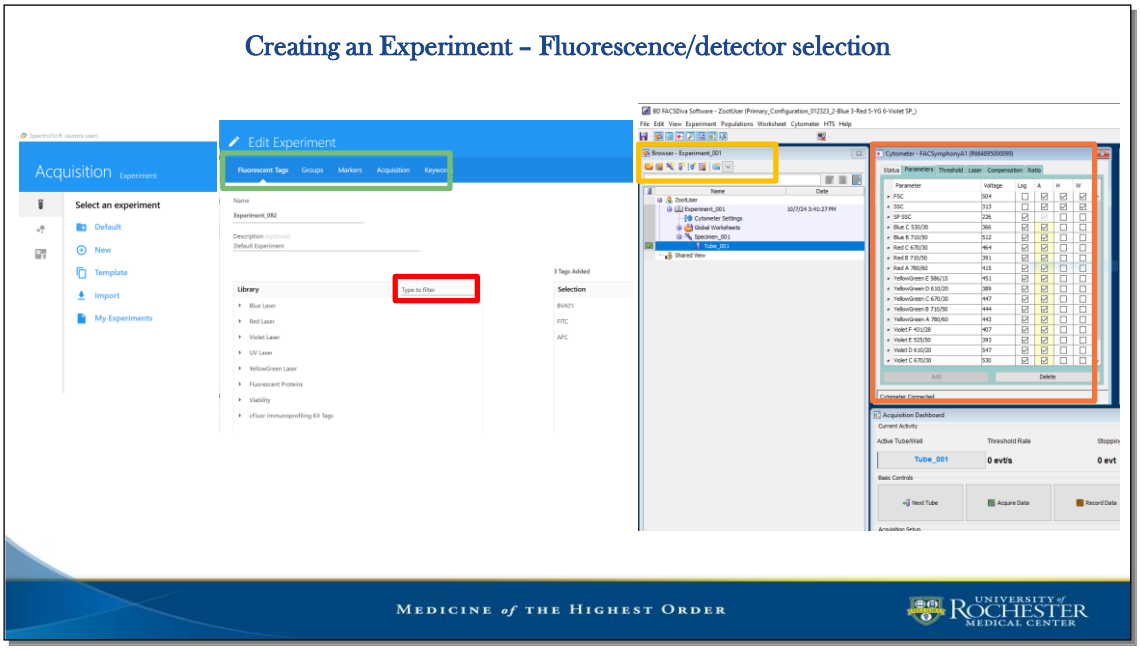
- Cytometer software typically contains these basic elements:
  - Experiments. Creating an experiment will likely be the first action taken when preparing to run samples on a cytometer.
  - Experiement layout/Experiment builder. This is the interface that allows a user to tailor the settings of the experiment to fit their specific needs.
  - Browser. The cytometer software will have a tube/sample browser that allows the user to create and select tubes where sample files will be recorded.
  - Acquisition Controls. These may be strictly in the software or split between the software and physical buttons on the face of the cytometer. These allow a user to start or stop sample acquisition and begin sample or control recording when ready.
  - Instrument Settings. These allow the user to adjust instrument settings such as detector gains or voltages.
  - Worksheet. Allows users to visualize the data from their recorded controls or samples. The worksheet provides a user with options to create plots and gates, and view statistics.



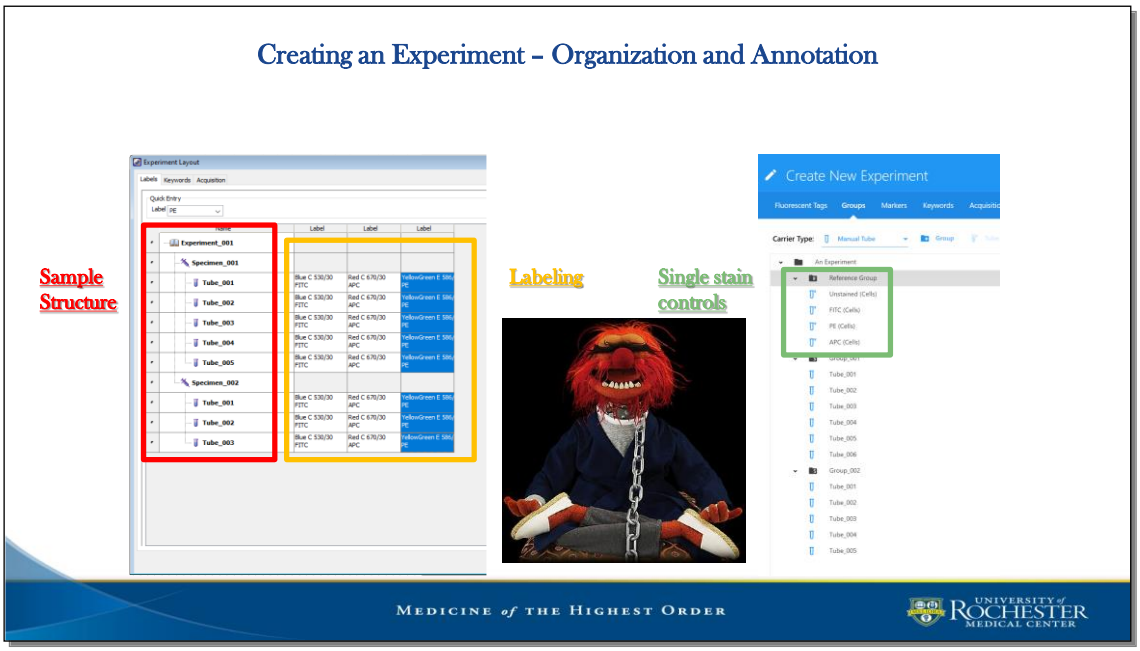


Creating an Experiment

- Before running samples on the cytometer an experiment needs to be created.
- Indicating which fluorochromes/detectors are being used in the experiment is the first crucial part of cytometer setup. This is necessary to ensure that signal is appropriately captured by the cytometer and visualized in the software.



- Sample structure and organization needs to be set up in the experiment.
  - The physical sample tubes being run on the instrument will correspond with individual tube elements in the software. Tubes can be made, deleted, and organized in the experiment at any point. 'Groups' or 'Specimens' can be used to group samples with other like samples. The degree to which samples tubes in an experiment are grouped depends on the complexity of experimental design and investigator preferences, though it is always recommended to have samples well organized and labeled.
  - The cytometer software will provide investigators with space to label their samples with information pertaining to what cell markers have been stained for. It is **critical** to apply accurate and informative labels for ease and reliability of data analysis.

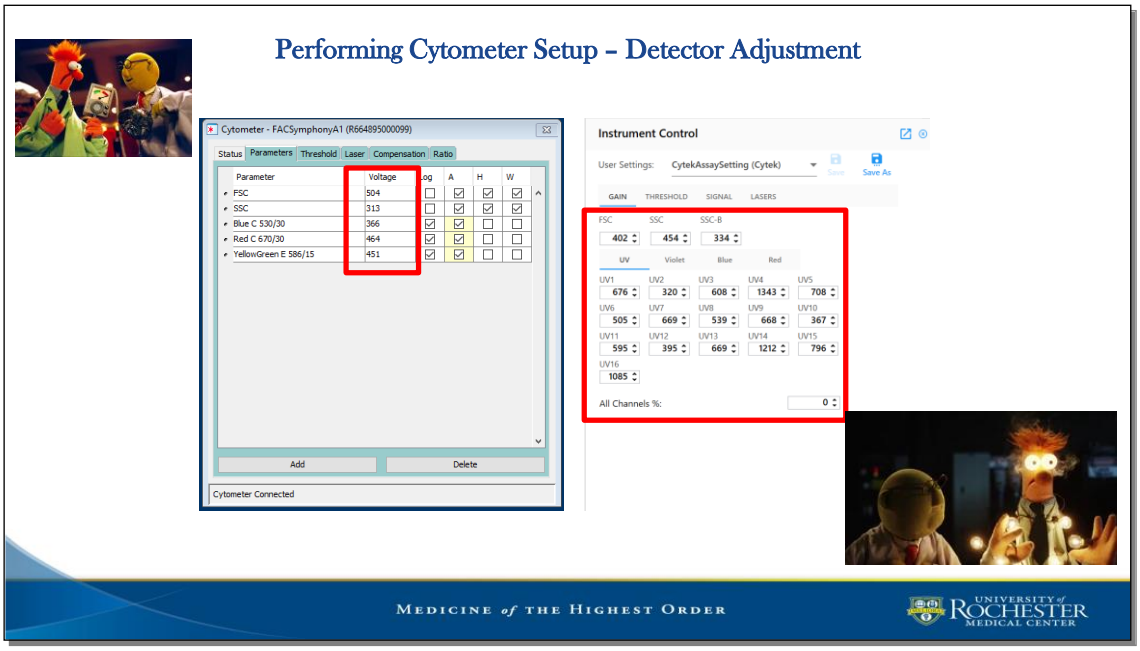


  - Additional keywords can be input for samples as well. Keyword information is embedded in the recorded files and can be accessed for analytical purposes using analysis platforms such as FCS Express or FlowJo. Again, it is always recommended to have good, clear annotation of samples.
  - The software will allow a user to apply and adjust various limits on sample recording
    - Depending on the platform being used, investigators can adjust the stopping criteria for recording their samples. These stopping criteria can include:
      - Number of events to record
      - Amount of time to record
      - Sample volume to uptake

- Creating single stain and unstained controls for compensation or unmixing
  - Single stain and unstained controls are required in most experiments.
  - When in doubt, it is better to have single stain and unstained controls in order to be able to correct for any spectral overlap that may occur between fluorescent elements in the panel. The cytometer software will allow investigators to create these controls based on the detectors or fluorochromes they have indicated will be used in the experiment.
  - More information on making these controls in the software and the rationale surrounding various choices an investigator can make while doing this will be covered in the instrument-specific training materials.

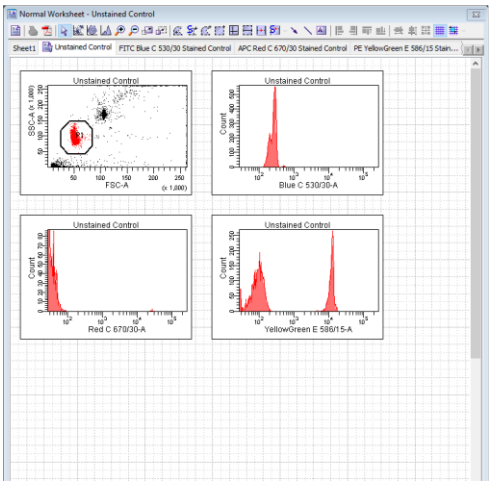
Performing Cytometer Setup

- Adjust scatter and fluorescence detectors
  - The next crucial step in the cytometer setup process is to check that detector voltages/gains are adjusted properly for the experiment, such that signal in scatter and fluorescence detectors is on scale.



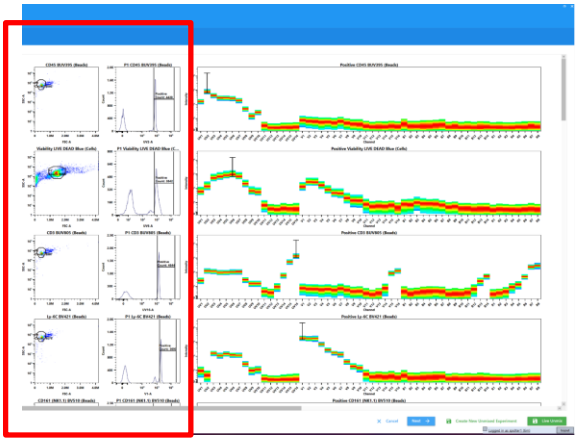
  - This should always be done while acquiring and viewing signal from controls, but <u>without recording.</u> Only once all detector voltages/gains have been verified to be well adjusted should an investigator begin recording controls or samples.
  - Fluorescence detector voltages/gains should not be adjusted once verified, however FSC and SSC voltages/gains can be adjusted as needed to be sure events are on scale.

Performing Cytometer Setup – Detector Adjustment/Recording Controls



- Record compensation or unmixing controls
  - Once voltages/gains are set, all unstained and single stain controls must be recorded in the corresponding tubes in the control group in the software.

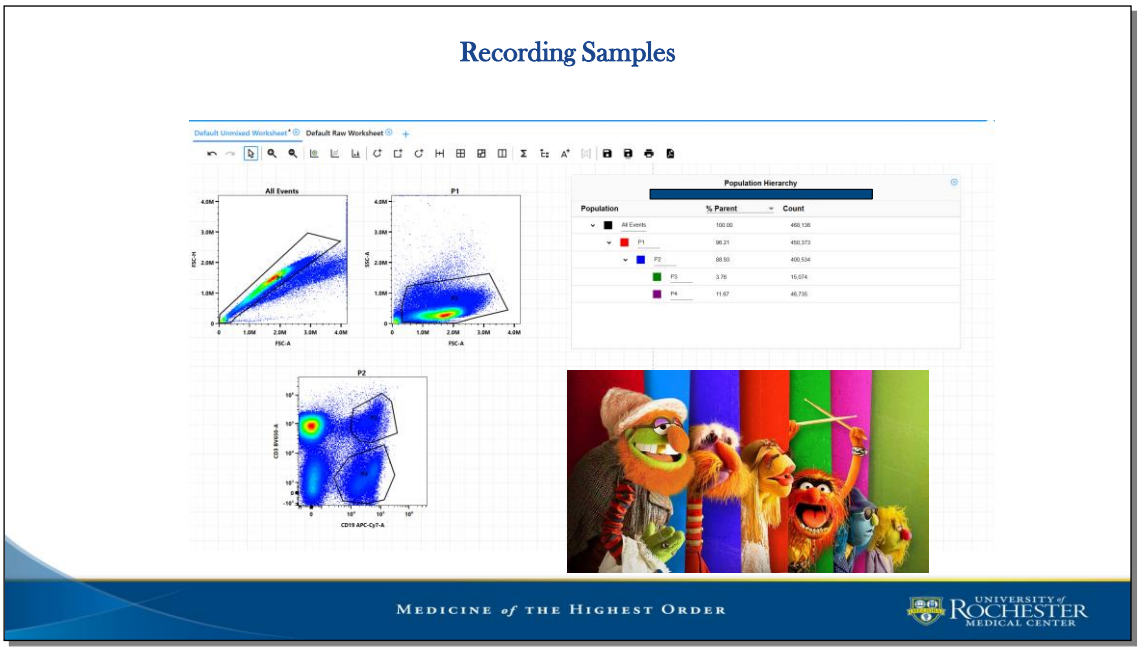
Performing Cytometer Setup – Recording Controls



- Calculate unmixing/compensation
  - After all controls have been recorded, compensation/unmixing can be calculated. At this step investigators should make sure that the positive or unstained signal is appropriately gated for each control.

Running Samples

- Build worksheet
  - At minimum a plot displaying FSC vs. SSC should be made on the worksheet before recording any samples. FSC and SSC voltages/gains often need to be adjusted when transitioning from recording controls to recording samples.
  - The worksheet interface allows the user to produce new plots, gates, statistics displays, and gating hierarchies. While final analysis of the data may not occur on the worksheet in the cytometer software, it is often helpful to build out the intended gating scheme for the samples being run so that cursory interpretation and analysis may be performed.



- Record samples.
  - It is important to make sure that the correct tube is selected in the software that corresponds with the sample to be run so the sample file is recorded in the right place with the right name.

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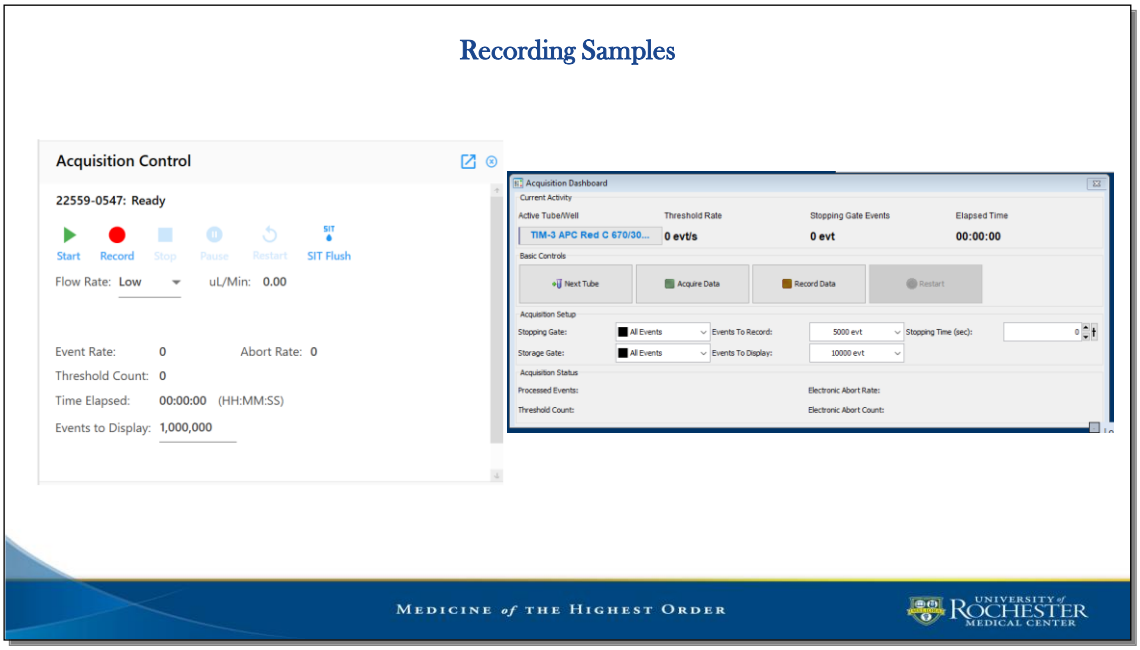

- It is not advisable to change the speed at which samples are being run (flow rate) while actively recording.
- Running samples at slower speeds results in less variation of recorded data.

- Save and export
  - The process of saving experiments and FCS files may be different for various instrument/software platforms. This will be covered in detail during instrument-specific training.
- After saving the experiment and FCS files, the instrument needs to be cleaned according to the instrument-specific cleaning protocol to be executed after each investigator's run is completed.