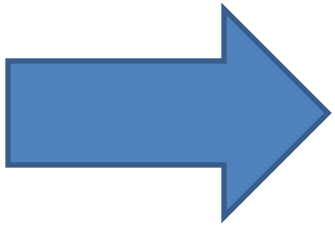
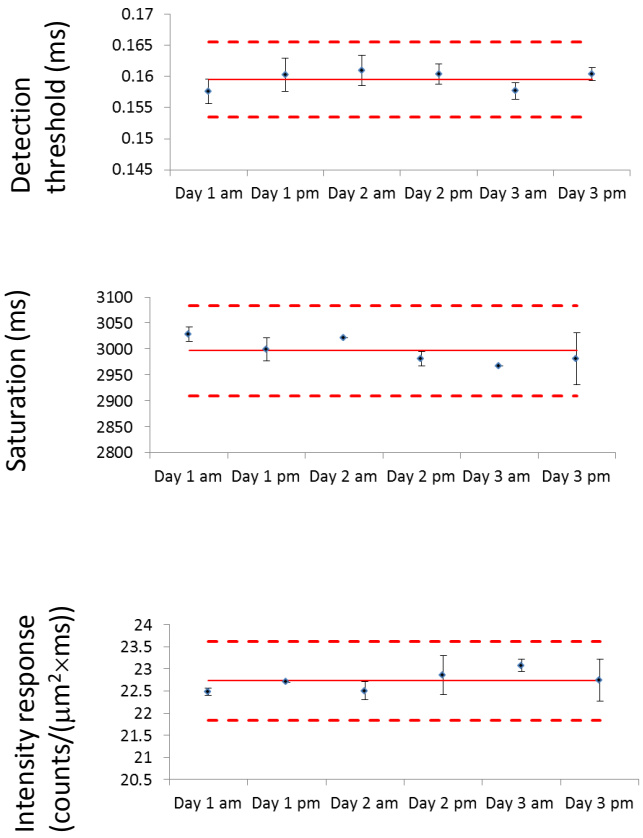
How to benchmark a wide field fluorescent microscope





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Summary

- The ability to generate measurements that characterize a wide-field fluorescence microscope is useful to ensure **nominal instrument performance**.
- The procedure described here **benchmarks** the microscope to a **commercial fluorescent glass** that has similar excitation and emission properties to FITC, GFP, etc.
- The procedure can be expanded to benchmark **other fluorescent channels** when suitable reference materials are available.

Limitations

- This is a beta-test
  - Only tested with ~10 different widefield microscope systems with CCD detectors (not CMOS) and 10x magnification objectives
  - GG 475 Schott glass fluorescence is excited between 450 nm-470 nm (lower wavelengths can cause photobleaching) and emits 520 nm – 700 nm

Step 1: Prerequisites

- Manual or automated wide field fluorescence microscope with 10x objective ~0.15-0.3 NA[1] and a CCD camera
- Filter set to image FITC or similar fluorophore.
- Ensure there is a Micro-Manager driver available for your microscope camera.

  -http://www.micro-manager.org/wiki/Device%20Support

  (tested with Roper CoolSnap FX, HQ, and HQ2; Zeiss AxioCam; ThorLabs DCC1545, Retiga 2000R)

[1]May also work with other objectives.

Step 2: Things you need

- Download
  - MicroManager (w/ImageJ) to the computer that will control the camera on the microscope (http://www.micro-manager.org/wiki/Download_Micro-Manager_Latest_Release)
  - MicroManager benchmarking script (http://www.nist.gov/mml/bbd/cell_systems/upload/Microscope_Performance_v1.2.txt)
- Identify a computer with a printer and Excel to use for control charting of benchmarking metrics
  - Download Excel spreadsheet template for control charting (http://www.nist.gov/mml/bbd/cell_systems/upload/Microscope_Charting Template_v2.xlsx)
- Schott Glass GG475 (25 mm dia x 3 mm thick)[1]
- Sample holder for the Schott Glass[2]
- Spatial calibration slide/stage micrometer

[1]Available from Edmund Scientific and other filter suppliers. Other fluorescent materials can be used for benchmarking. The material should be photostable and homogenously fluorescent to facilitate accurate benchmarking.

[2]See next page.

Example Sample Holders

Universal stage adapter

35 mm dish

Schott Glass GG475

10 cm dish

6 well plate

Hole in polystyrene sample holders was cut with a Dremel tool. Rough edges were removed by scraping with a razor blade.

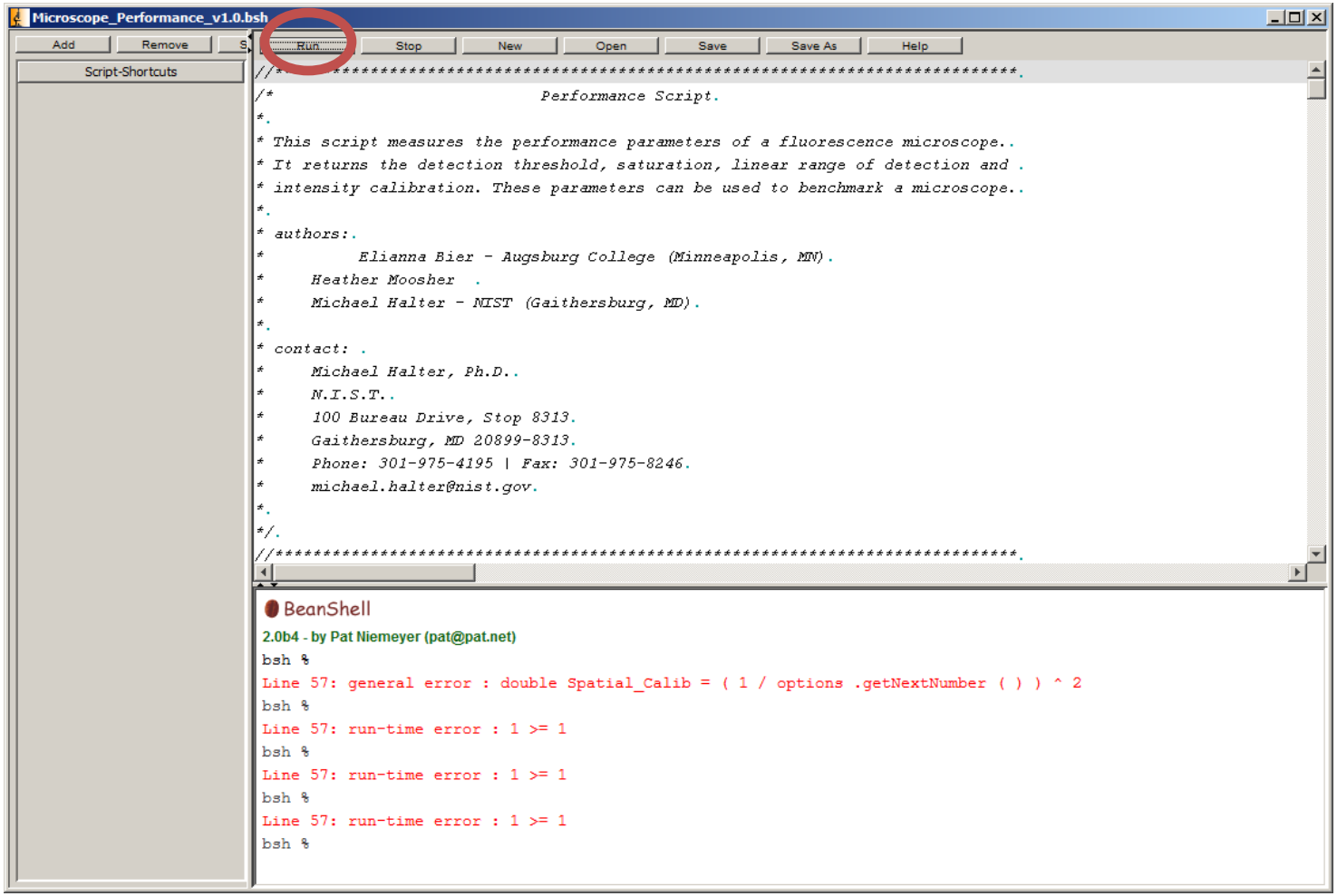
Step 3: Install and Start MicroManager

(skip this step if you already run MicroManager)

- Turn on camera and microscope. Set microscope filters to FITC (or similar filter set) and place 10x objective under specimen location.
- Install MicroManager using instructions at the MicroManager website (http://www.micro-manager.org/wiki/Micro-Manager_User%27s_Guide#Installation).
- Start MicroManager.
- Configure MicroManager to control your microscope camera
  - If you have not previously installed MicroManager:
    - Tools > Hardware Configuration Wizard > Create new configuration
    - Select the camera and press Add
    - Accept the remainder of settings using defaults values
- Place fluorescent glass on the microscope stage
- Test microscope operation by starting live acquisition and make sure you can capture fluorescent images of the Schott glass. TURN OFF LIVE ACQUISITION MODE

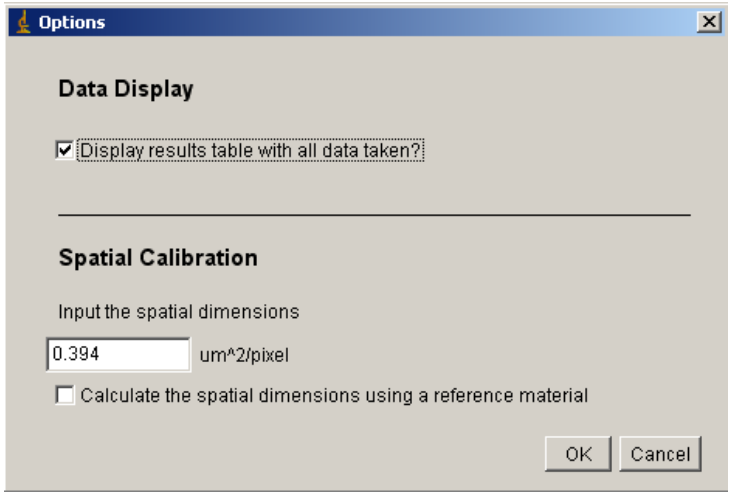
Step 4: Install and Run Benchmarking Script

- From MicroManager menu
  - Tools > Scrip Panel > Open (Microscope_Performance_v1.1.bsh)
  - Run



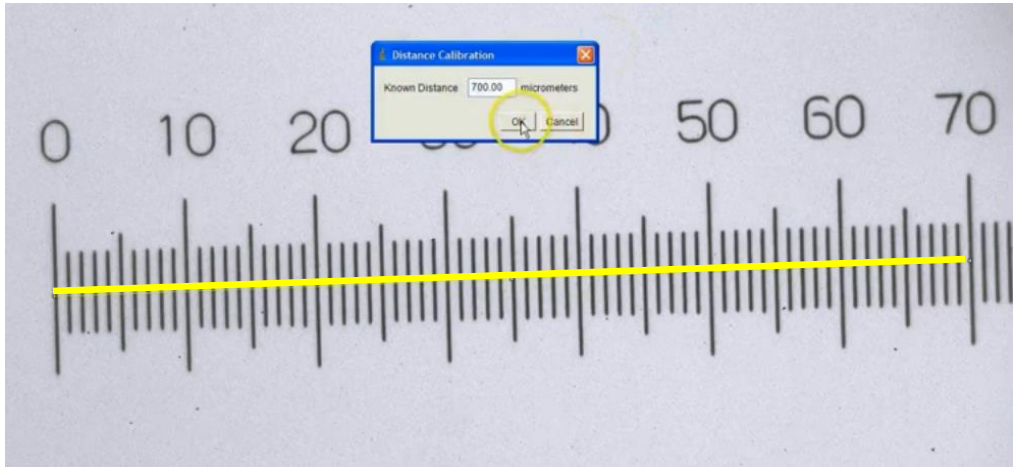
Step 5a: Data Display and Spatial Calibration Window

- Click Data Display checkbox.
- If the spatial calibration is known, the value can be entered in text box. If not, check the box indicating that you will generate a calibration.
- Press OK.



Step 5b. Spatial Calibration

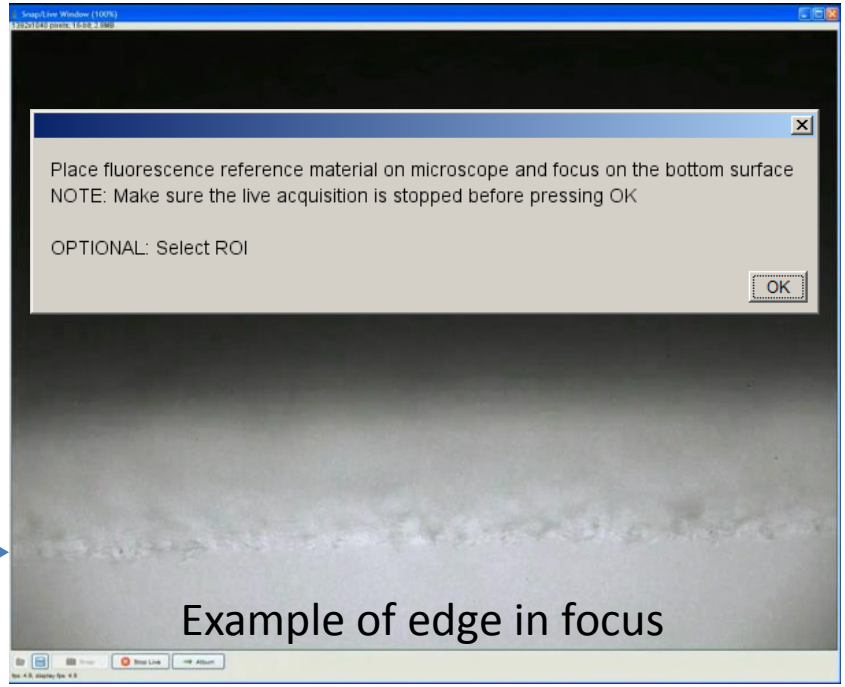
- Place the spatial calibration slide on the stage and focus. Then, press OK.
- Draw a line between two calibration points then click OK.
- In the next dialog input the distance of the line in micrometers



Step 6. Insert Fluorescent Glass Sample and Focus

- Place the sample holder and glass reference material on the sample stage.
- Set the focus

1. Move the stage so that the edge of the fluorescent glass in in the field of view.
2. Focus on the edge of the fluorescent materials proximal to the microscope objective.
3. Move the stage to a position where the field of view is filled with the fluorescent reference material.

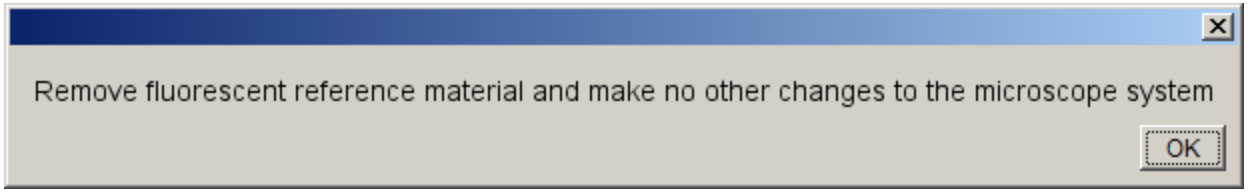


Example of edge in focus

- Click ‘OK’ on the open dialog box

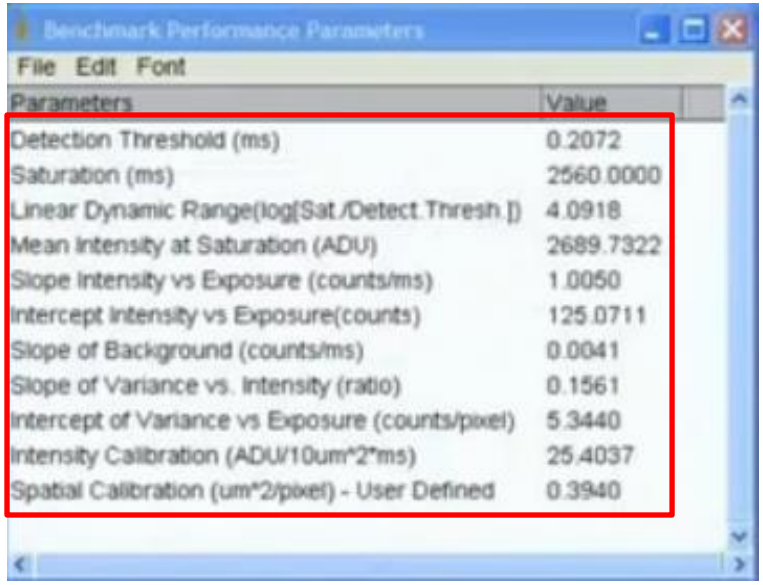
Step 7: Setting up background fluorescence measurement

- Carefully remove Schott glass from the sample holder and store in a safe place
- Make no other changes to the microscope system and click ‘Ok’ in the open dialog box



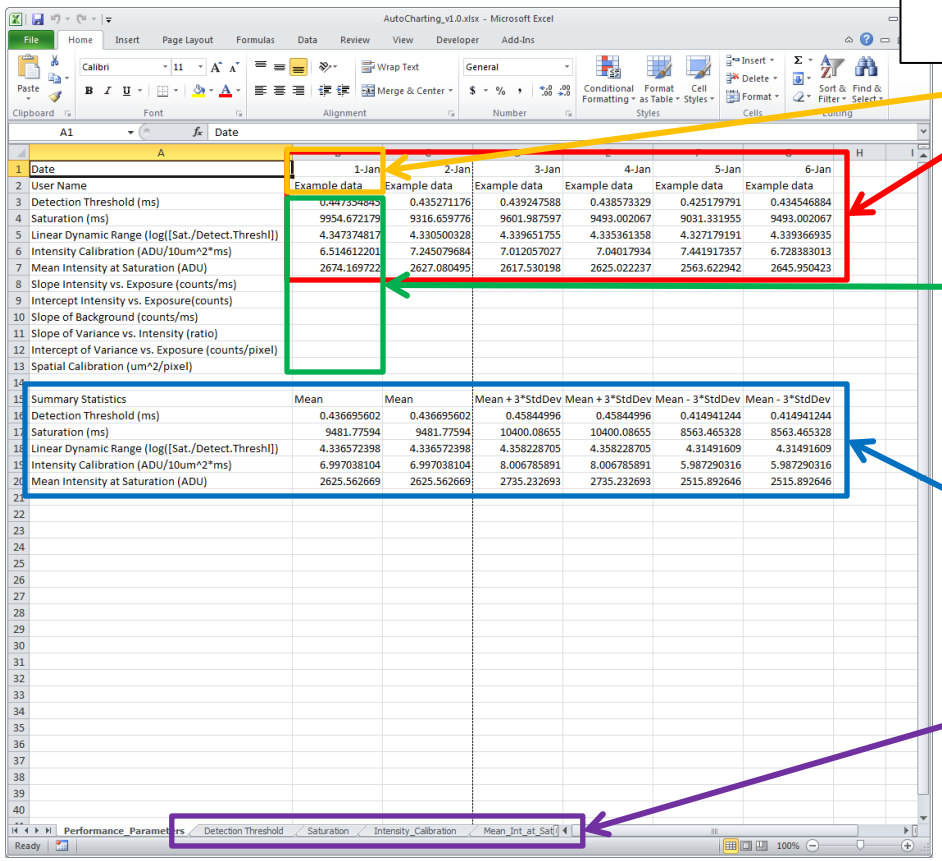
Step 8: Charting

- Copy the data from the Benchmark Performance Parameters table of results
  - Ctrl-A to select all
  - Ctrl-C to copy



Step 8: Charting

- Open Charting Template in Excel
  - If using for the first time, delete example data from the Charting Template
- Paste the Benchmark Performance Parameters into the appropriate column
- You will need to remove the column with the parameter descriptions that is automatically copied from the MicroManager results table.



Add Date and User Name with each set of benchmark data.

Delete example data

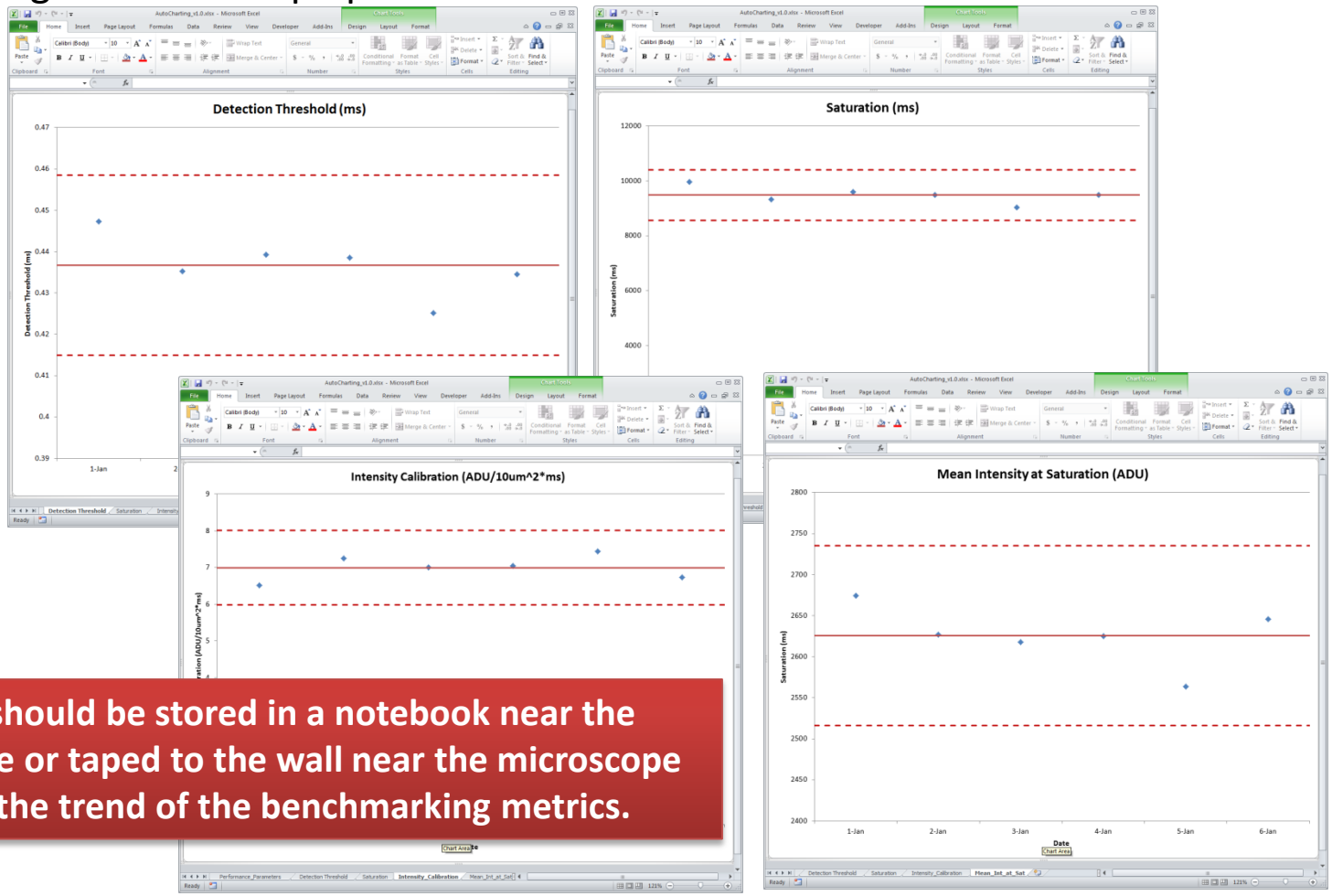
Record new Benchmark Performance Parameters in appropriate column.

Data and statistics used for charting (**DO NOT CHANGE**).

Each Chart is **automatically updated and stored** as separate Excel Chart Sheet.

Step 8: Charting

- **Detection Threshold**, **Saturation**, **Intensity Calibration**, and **Mean Intensity at Saturation** are critical metrics that should be charted to ensure nominal microscope operation.
- Each plot should **automatically update** when new numbers are recorded.
- Save Excel file with charting data. Plots should be **printed each time new data is entered** for evaluating the microscope performance.



The plots should be stored in a notebook near the microscope or taped to the wall near the microscope to display the trend of the benchmarking metrics.

Brief Definitions of Charted Parameters

- **Detection Threshold[1]:** This is a measure of the sensitivity of your microscope to detecting low levels of light. It is the shortest possible exposure time when the SNR of fluorescence emitted from the glass is greater than or equal to 3.
- **Saturation[1]:** This is a measure of when your detector response becomes non-linear. It is the exposure time at which the pixel variance plotted against intensity becomes non-linear.
- **Intensity Calibration[1]:** This can be used to calibrate for day-to-day variations on your microscope. It is the counts/(10 $\mu m^2 \times s$) detected from the fluorescent glass used as a reference.
- **Mean Intensity at Saturation[1]:** This is a measure of when your detector response becomes non-linear. Similar to "Saturation", but provided in units of "counts" on the detector. In general, intensity measurements should be below this value to remain in the linear range.

[1]A more in-depth description of these parameters and how they are determined is available in the publication, "Performance Benchmarking and Intensity Calibration of a Widefield Fluorescence Microscope Using Fluorescent Glass" Cytometry A, in review (2014)

Interpreting Changes in Charted Parameters

- Detection Threshold, Saturation, and Intensity Calibration all increase or decrease proportionally
  - Causes: Illumination source fluctuation or a beam splitter or neutral density filter placed in light path
- Detection Threshold only changes
  - Indicates a change in the noise floor of the detector. Could be from changing the read rate setting on detector, the temperature of the detector or degradation of the sensor components
- Other combinations of changes
  - The parameters will likely change when the optical components of the microscope or settings on the detector are changed, sometimes in non-obvious ways. Changes in the parameters in the absence of any hardware or software changes indicates drift in the measurement system.