2023 iGEM InterLab Study

Derived from: InterLab 2022 - Calibration Protocol_v2

Automated multicolor fluorescence per particle calibration protocol

Many iGEM projects rely on measurements of cells containing fluorescent proteins. Most of these measurements are performed on versatile, 96-well plate readers Plate readers report fluorescence values in arbitrary units that vary widely from instrument to instrument. Thus, absolute fluorescence values cannot be directly compared from one instrument to another, and therefore, the results of one iGEM project are hard to assess relative to a related or earlier project. In order to more accurately compare fluorescence output of biological devices, it is necessary to construct a standard fluorescence curve using reference materials. This modification of the original iGEM protocol uses two replicates of three dyes of different color to assess fluorescence, as well as nanosphere beads to estimate cell density. The current protocol is adapted from:

- https://dx.doi.org/10.17504/protocols.io.bht7j6rn
- https://dx.doi.org/10.17504/protocols.io.6zrhf56.

Protocol in short: Three different reference materials (dyes in solution) will be used to calibrate fluorescence, and suspensions of silica nanoparticles will be used to estimate cell density. Serial dilutions from stock solutions / suspensions, at known initial concentrations, will be used to construct a calibration curve. The protocol utilizes 96-well plates. After making stocks, serial dilutions will be made by adding 200uL of each solution in the first columns of the plate, and then aspirating 100uL from one well and adding the aliquot to the corresponding well in the next column that contains 100ul buffer. Mixing is achieved by pipetting up and down three times, and then a new, 100ul aliquot is transferred into another well containing buffer in the next column. The process is repeated until the 11th dilution is performed, when the last 100uL aliquot is simply discarded. When finished, the wells should contain serially diluted 200ul solutions/suspensions for calibration experiments.

Important note: For the calibration and your experiments you must use the same type of plates that provide for the same volumes. You must also use the same instrument settings (e.g., filters or excitation and emission wavelengths) for the calibration and for the experiment. If you do not use the same plates, volumes, and settings, the measurements will not be valid.

Protocol Outputs:

- `fluorescein and bead fluorescence measurements of calibration plate`
- `sulforhodamine 101 fluorescence measurements of calibration plate`
- `cascade blue fluorescence measurements of calibration plate`
- `NanoCym 950nm monodisperse silica nanoparticle absorbance measurements of calibration plate`

Protocol Materials (from the Measurement Kit)

- Fluorescein (0.1 umoles dried down); Extinction coefficient = xx,xxx M-1 cm-1 or y,yyy cm2 g-1
- Cascade Blue (0.1 umoles dried down); Extinction coefficient = M-1 cm-1 or cm2 g-1
- Sulforhodamine 101 (0.02 umoles dried down); Extinction coefficient = M-1 cm-1 or cm2 g-1
- NanoCym 950nm monodisperse silica nanoparticles (3e9 beads/mL)

Protocol Materials (provided by the Team)

- Water, sterile-filtered, BioReagent, suitable for cell culture
- Phosphate Buffered Saline 1X (pH 7.4)
- 96 well plate black with transparent flat bottom
- Foil-covered or amber tubes.
- Pipette for 1 mL (single)
- Plate reader able to measure absorbance at 600nm and fluorescence either with filters or monochromator.
- Opentrons OT-2
- OT-2 Pipette P300 (Gen 2) or OT-2 Pipette P1000 (Gen 2) [Link]
- Tip rack OT-2 compatible
- Tube rack OT-2 compatible (tested labware: aluminum block)

Protocol Steps:

Making the stock and working solutions

Note: Resuspend all the fluorescent calibrants in amber or foil-covered tubes and do not leave them out in the light for too long, as they are light sensitive.

Fluorescein

1. Provision the stock reagent tube with Fluorescein calibrant from the Measurement Kit. This is a powder. Spin down to make sure the pellet is at the bottom.
2. Transfer 1.0mL of Phosphate Buffered Saline (PBS) to stock reagent tube Fluorescein calibrant. Resuspend pipetting up and down a few times, and vortex 30 seconds. This is now the reconstituted Stock Fluorescein Solution 10X with an expected concentration of 100 uM in PBS. (The exact concentration can be ascertained by taking an absorbance spectrum of a series of dilutions and using the extinction coefficient to calculate concentration.)
3. Prepare a tube to make the working concentration solution 1X Fluorescein.

4. Dilute the Stock (~100 uM Fluorescein Solution 10-fold with 1X PBS to make a 1X reference working solution with an estimated concentration of 10 uM (e..g. dilute 100 uL of 10X fluorescein stock into 900 uL 1X PBS.)

Sulforhodamine 101

5. Provision the stock reagent tube containing Sulforhodamine 101 calibrant from the Measurement Kit. This is a powder. Spin down to make sure the pellet is at the bottom.
6. Transfer 1.0mL of PBS to stock reagent tube Sulforhodamine 101 calibrant. Resuspend pipetting up and down a few times, and vortex 30 seconds. The reconstituted Stock Sulforhodamine 101 Solution should have a final concentration of 20 uM in PBS. (The exact concentration can be ascertained by taking an absorbance spectrum of a series of dilutions and using the extinction coefficient to calculate concentration.)
7. Prepare a tube to make the working concentration solution 1X Sulforhodamine 101.
8. Dilute the Stock Sulforhodamine 101 Solution 10-fold with 1X PBS to make a 1X reference working solution with an estimated concentration of 2 uM (e.g. dilute 100 uL of 10X Sulforhodamine 101 stock into 900 uL 1X PBS.)

Cascade Blue

9. Provision the stock reagent tube containing Cascade blue calibrant from the Measurement Kit. This is a powder. Spin down to make sure the pellet is at the bottom.
10. Transfer 1.0mL of Water to stock reagent tube Cascade Blue calibrant. Resuspend pipetting up and down a few times, and vortex 30 seconds. The reconstituted Stock Cascade Blue Solution 10X should have a final concentration of 100 uM in water. (The exact concentration can be ascertained by taking an absorbance spectrum of a series of dilutions and using the extinction coefficient to calculate concentration.)
11. Prepare a tube to make the working concentration solution 1X Cascade Blue.
12. Dilute the Stock Cascade Blue Solution 10-fold with water to make a 1X reference working solution with an estimated concentration of 10 uM (e.g. dilute 100 uL of 10X Cascade blue stock into 900 uL water.)

NanoCym 950nm monodisperse silica nanoparticles

13. Provision the stock reagent container containing NanoCym 950nm microspheres
14. This tube contains 850uL of NanoCym 950nm monodisperse silica nanoparticles with a concentration of 3e9 microspheres/mL in Water.
15. Vortex NanoCym 950 nm microspheres, they are ready to be used.

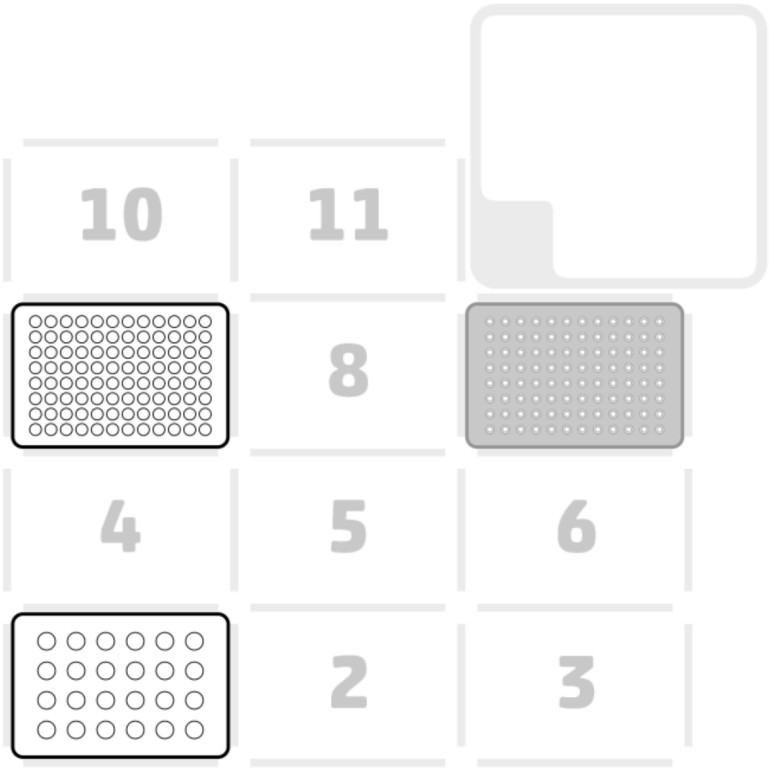
At this stage it would be helpful to display a calculation that relates how one could weigh out the nanoparticles and determine the number of particles present based on the density of the particles.

Calibration Protocol

Set the OT-2 deck

16. Place the tip rack in deck slot 9

17. Place the tube rack in deck slot 1
18. Place the 96 well plate in deck slot 7



Your deck should look like this.

Set the OT-2 tube rack

19. Place Fluorescein 1X in tube rack slot A1
20. Place Sulforhodamine 101 1X in tube rack slot A2
21. Place Cascade blue 1X solution in tube rack slot A3
22. Place NanoCym 950nm in tube rack slot A4
23. Place 1.5 mL tubes with 1.3 mL PBS in tube rack slots B1, B2, B3 and B4
24. Place 1.5 mL tubes with 1.3 mL water in tube rack slots C1, C2, C3 and C4
25. Close the OT-2 door

A1: Sulforhodamine_1x	A2: PBS	A3: Water			A6: Bin
B1: Fluorescein_1x	B2: PBS	B3: Water			
C1: Cascade_blue_1x	C2: PBS	C3: Water			
D1: Microspheres_1x	D2: PBS	D3: Water			

Your tube rack should be like this.

Run the script

26. Open the Opentrons App in the computer connected to the OT-2
27. Load the script (run_iGEM2022_rgb_od_libre.py) in the Opentrons App
28. Run the script/protocol using the Opentrons App

Measurement

29. Measure Fluorescein fluorescence of calibration plate (wells A1:B12) with excitation wavelength of 488.0nm and emission wavelength of 530.0nm and 30.0nm bandpass.
30. Measure Sulforhodamine 101 fluorescence of calibration plate (wells C1:C12 and D1:D12) with excitation wavelength of 561.0nm and emission wavelength of 610.0nm and 20.0nm bandpass.
31. Measure Cascade blue fluorescence of calibration plate (wells E1:E12 and F1:F12) with excitation wavelength of 405.0nm and emission wavelength of 450.0nm and 50.0nm bandpass.
32. Measure NanoCym 950nm monodisperse silica nanoparticles absorbance of calibration plate (wells G1:G12 and H1:H12) at 600.0nm.
33. Import data for fluorescein fluorescence, sulforhodamine 101 fluorescence, cascade blue fluorescence, and absorbance measurements into the provided Excel file.

    Note: If your equipment does not have facility for choosing the specified wavelengths or bandpass filters, choose the nearest one(s) and make note of the parameters. This information will be requested in the data uploading process.