**PROTOCOL** accompanying the manuscript “Characterization of Aggregate Load & Pattern in living Yeast Cells by Flow Cytometry”

**Yeast Culturing**

**Equipment:**

* Plate shaker (30°C)
* Spectrophotometer capable of measuring optical density (OD) at 600nm
* 10 x 4 x 45mm disposable polystyrene cuvettes
* 15ml falcons
* 12 x 75 polypropylene tubes
* Centrifuge at room temperature (20°C).

**Reagents:**

* Yeast rich media (YPD)
* Yeast selection media (SD-ura)
* Phosphate-buffered saline (PBS)
* 0.5M Ethylenediaminetetraacetic acid (EDTA)
* Fetal calf serum (FCS)

8% paraformaldehyde (PFA) in PBS; Caution: paraformaldehyde is a known carcinogen. Avoid inhalation, ingestion, or contact with skin, eyes, or mucous membranes.

**Reagent Setup:**

* **Resuspension buffer.** Mix 50ml of FCS, 5ml of 0.5M EDTA with 445ml of PBS. Store at 4°C.

**Procedure:**

**Strains preparation:** TIMING: 18 hours

1. Grow cells expressing the fluorescently tagged protein substrate of interest, in a pre-culture of selective/non selective liquid media (≤ 20ml) for 16-18hrs at 30°C with shaking (150 rpm).
2. Measure OD of cell suspension and dilute to an OD of 0.15 in fresh media (≤ 20ml).
3. Continue to culture cells at 30°C with shaking until a logarithmic growth phase is reached; OD 0.3-0.8 (*ca.*3hrs).

CRITICAL STEP: Do not let cells grow above an OD of 1.0; aggregation of the protein substrate will be affected when cells reach a saturated growth phase.

**Sample preparation:** Analysis can be performed with living cells (Option A) or with PFA-fixed cells (Option B); ethanol-based fixation should be avoided as it can affect the integrity of the aggregates. TIMING: non-fixed cells 15 minutes/ fixed cells 45 minutes

**A.** **Living cells**

1. Transfer 10ml of the logarithmic culture into 15ml tube and centrifuge at 4000g for 6 minutes at room temperature.
2. Aspirate and discard the supernatant and gently resuspend in 1ml of resuspension buffer
3. Transfer the cell suspension to an appropriately labelled tube and store at room temperature, protected from the light.

CRITICAL STEP: Cells should be analysed within 1hr after collection of the cells.

**B. Fixed cells**

1. Transfer 10ml of the logarithmic culture into 15ml tube and centrifuge at 4000g for 6 minutes at room temperature.
2. Aspirate 5ml of the supernatant and add 5ml of 8% PFA.
3. Vortex thoroughly and then incubate at room temperature for 10 minutes.
4. Centrifuge at 3000g for 5 minutes at room temperature.
5. Aspirate and discard the supernatant and wash the cell pellet twice in 5ml of PBS (3000g/5mins/room temperature). Optional: Cell pellet can be resuspended in 1ml of PBS and stored at 4°C for ≤ 3 days.
6. Resuspend in 1ml of resuspension buffer and transfer the cell suspension to an appropriately labelled tube and store at room temperature, protected from the light.

**Analysis of Samples by Flow cytometry:**

**Equipment:**

* Flow cytometer, equipped with 488nm laser for excitation and appropriate filters for GFP/FITC); BD Canto or an equivalent
* FlowJo version xV0.7 (*OR, USA*).
* Graph pad Prism 6.05 (*GraphPad Software, San Diego California USA*)

**Setup of Flow Cytometer & Running of Samples:** TIMING: 30 minutes

1. Ensure that the flow cytometer is equipped with the appropriate laser and filter sets to measure the fluorescently tagged protein of interest. This can be determined under the default cytometer configuration on the system. In the case of GFP, this would be 488nm laser for excitation and FITC/GFP channel for detection.
2. The flow cytometer must be able to collect the height, area and weight parameters for the channel of interest. For the BD Canto, these parameters can be selected under the Inspector – Cytometer menu. The GFP signal is best detected with logarithmic amplification
3. Select an appropriate flow rate for the samples. This parameter can vary depending upon the system used and should be determined empirically. For the BD Canto, a low (10µl/min) flow rate was selected.
4. Select the total number of events/cells to be collected per sample. The minimum number of events should be 50,000.
5. Create the following scatter plots under global worksheet:
   * Side scatter (SSC-A) versus forward scatter (FSC-A)
   * GFP-A versus FSC-A
   * GFP-H versus GFP-W
6. Create a statistical view that will display the mean fluorescent intensity (MFI) for the selected GFP parameters.
7. Set the voltage of the FSC-A and SSC-A channels, such that the smallest cells fit into the lower left 10% of the scatter plot. No forward scatter threshold is required, since all events from the culture were confirmed by microscopy to be intact and viable cells.
8. Determine the background fluorescence and minimum sample fluorescence. To set the photomultipliers (PMTs) of the fluorochrome of interest, a positive control exhibiting the fluorescence of choice and a non-fluorescent negative control are required. Using the GFP-A vs. FSC-A scatter plot the voltage of the GFP channel is adjusted so that the cells of the negative control fall within the lower quarter of the scatter plot (≤ 103 GFP-A). A rectangular gate (P1) is then created above this region to define those cells that are GFP positive. This P1 gate is referred to as the *parent* and all cells t
9. Once the flow cytometer has been setup, as described above, the samples can be run. Mean running time per samples is *ca.* ≤ 10secs on BD Canto with the stated settings.
10. After the run, the samples can either be discarded or taken for further analysis by microscopy.

CRITICAL STEP: Yeast is a microorganism, it is therefore critical after running samples in the flow cytometer that a thorough cleaning of the system is performed to ensure that there is no residual sample left in the system leading to contamination of the fluidics. This is particularly relevant when working with live yeast. Users should discuss the best strategies for cleaning with the head of flow cytometer core facility.

**Optional: Sizing of the subpopulations using calibration beads:** TIMING: 15 minutes.

The intensity of the FSC signal is proportional to a particle’ size. It is, therefore, possible to measure the size of the cell population by comparing the FSC signal to a population of microspheres of known size(s). This type of measure is semi-quantitative as a cell’s FSC signal is not only related to size but to other factors, such as the refractive index. As the refractive index of the cell may differ to that of them microspheres, the sizes estimated by this method may not be the actual size of the cell.

1. Use 2-5 drops of each SPHEROTM Nano Polystyrene Size Standards (or similar) and dilute them into water
2. Run the diluted beads and define gates using the histogram plot
3. One you have defined all the gates, apply them to the different plots from your samples, in order to give a specific size or size range to your populations or subpopulations

**Optional: Pulse Shape Analysis of Samples:** Pulse shape analysis is performed as previously described[12](#_ENREF_12). TIMING: 1-2 hours

1. Export the FCS files for the P1 gate of each sample, selecting only the GFP-W read-out.
2. Convert the FCS files into Microsoft Office Excel-delimited text file. This can done using either FlowJo or FCS Extract 1.02 Software (<http://research.stowers-institute.org/efg/ScientificSoftware/Utility/FCSExtract/>)
3. Open files in GraphPad Prism, using the column worksheet. Analyse the frequency distribution using the appropriate tool (Column analysis > frequency distribution), tabulating the data/plotting as relative frequency in percentages.
4. Perform non-linear regression on the frequency distribution data, fitting to a Gaussian curve, to determine amplitude, SD and mean GFP-W signal. For replicates, export the values for these parameters to a new file for further analysis. Comparison of these parameters between different cells/aggregate phenotypes is performed using a student’s t-test (two groups) or one-way ANNOVA (greater than two groups).

**Troubleshooting**

**Fixation of Yeast:**

The protocol can be applied to living cells as well as to fixed cells. However, the fixation procedure can have an impact on the aggregates. Ethanol fixation significantly affects the integrity of the aggregates leading to their complete loss. Fixation with paraformaldehyde does not greatly affect larger aggregates; however, smaller aggregates can become no longer visible by microscopy after PFA fixation.

**Flow Cytometry:**

The most critical factor for ensuring that this protocol is successful is the setup of the flow cytometry. The system must be equipped with the appropriate laser(s) and filter(s) setting to measure the fluorescently tagged protein of interest. If this is overlooked, then sub-optimal results will be obtained. Most basic flow cytometry system, such as BD FACScan, only consists of one blue (488nm) argon laser from which three different colours (FL1/Green, FL2/Orange and FL3/Far-red) can be detected. Such a system would therefore be suitable for the detection of GFP and YFP-tagged proteins only. The laser and filter settings for your system can be determined under the default cytometer configuration on the system (*Refer to the user manual*). A summary table of the five major fluorescent protein tags is shown in Table 1, including optimal lasers and filters.

It is highly recommend that prior to any comprehensive analysis, such as high-through put screening, a sample of the culture with and without the fluorescently tagged protein of interest should be run on the flow cytometry as to determine whether the system is suitable.

If detection is not possible, despite the appropriate lasers and filters being available, then the optical alignment of the system may sub-optimal. This can be determined and corrected by using quality control beads. These microsphere beads with known sizes and/or fluorescence levels, allow for the system to automatically characterize, track and report measurements of the laser(s). Any changes in the amount of voltage for a particularly fluorophore, required to achieve the same intensity, would indicate a loss of laser power, particularly if it is across all detectors for a given laser. If it is isolated to a single fluorophore, it could indicate that the detector has failed. In addition, if the coefficient of variation of the bead population increases, such as with respect to fluorescence intensity, then it would indicate that the sensitivity of the system has decreased and would suggest that there is either a misalignment of the laser or an issue with detection optics. If this is the case, then the manufacturer of the system should be contacted. If detection is still not possible, then the issue is likely due to the expression level of the construct and/or fluorescent tag chosen.

**Table 1.** Commonly used fluorescent protein tags [Methods. 2012 Jul;57(3):318-30 & Methods Mol Biol. 2016;1369:309-46]

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fluorescent Protein** | **Colour** | **Brightness** | **ExMAX (nm)** | **EmMAX (nm)** | **Recommend Laser (nm)** | **Recommend bandpass filters (nm)** |
| EGFP | Green | 33.6 | 484 | 507 | 488 | 510/21, 530/30 |
| EYFP | Yellow | 50.9 | 514 | 527 | 488, 505, 514, 517, 532 | 530/30, 550/30, 556/21, 575/26, 585/42 |
| ECFP | Cyan | 13.0 | 439 | 476 | 405,440,457 | 450/50, 460/50, 485/22 with 488 nm block |
| DsRed | Short-red | 3.5 | 553 | 583 | 532, 550, 561 | 585/42, 590/20, 610/20 |
| mCherry | 15.8 |