Monitoring Yeast Counts, Viability, and Metabolic Activity in Brewing with Orflo's Moxi GO II – Next Generation Flow Cytometer

Introduction/Background

History

The documented history of brewing dates back over 5,000 years and is generally thought to have transpired for several thousand more years before that.[1,2] Regardless of the exact inception time, brewing has grown into a global industry with a staggering estimated market value of $530 billion (USD, 2016).[3] Despite its long history, the process of making beer remains a highly nuanced one in its goal of achieving a consistent result from a deceptively complex biological system. The technique requires the management of numerous variables related to environmental conditions, ingredients, and general recipes/protocols. Not surprisingly, given the size of the market and complexity of the process, over 440,000[4] scholarly articles have been dedicated to brewing in order to better understand the contributing factors with the overall goal of improving both quality and consistency of the end result.

Figure 1 – Orflo's Moxi GO II – Next Generation Flow Cytometer. Image shows user loading a sample into the two-test disposable flow cell. The Moxi GO II is a configurable flow cytometer with a 488nm laser and up to two fluorescence recording channels (2 PMT's with emission filters at 525/45nm filter and 561nm/LP, respectively).

Importance of Yeast Counts/Viability

At the core of the brewing process is the conversion of sugar into alchohol by yeast. Beyond the initial selection of the yeast strain, the understanding of the shifting characteristics of the yeast in the wort, relative to the constantly-changing environmental conditions, is critical. At a bare minimum, brewers need to maintain proper concentrations of yeast throughout the process by adding, or "pitching", yeast at various timepoints. Ensuring the correct pitching of viable yeast cells into the wort can have pronounced effects, with under-pitching resulting in longer fermentations (and costs associated with the loss in time and throughput) and over-pitching result in yeast death, filtration issues, and taste issues.[5] The simplest approach to pitching ignores yeast viability by simply adding a fixed amount (weight or volume) of yeast slurry per liter of brew. However, aside from the smallest craft/home brewers, most brewers have come to realize the importance of exact yeast cell counts and viability measurements in the achievement of batch-to-batch repeatiblity.

Yeast Cell Counting and Viability Technology

One commonly used approach to counting yeast cells involves the use of a microscope with a hemocytometer, a glass chamber with a precisely calibrated fluidic volumetric regions. The hemocytometer allows for the conversion of manual/visual counts into cell concentrations and the determination of cell viability through staining, typically using methylene blue. Hemocytometers in brewing gained traction due to the relatively low initial cost. But, the approach is notoriously inaccurate and highly variable in its output with issues such as: variable user interpretation and count consistency discrepancies, chamber loading biases, focusing-induced variability, and statistically small sample sizes. Recently, attempts have been made to automate that process through software in the form of "imaging system" counters. However, those systems are fundamentally flawed in that they inherited the weaknesses of the underlying hemocytometer approach and have even exascerbated them as they attempt to rigidly apply automated rules to

analyzing complex 2D images of cells. Specifically, the small size of the yeast challenges the resolution of the imaging system software as "debris" particles in the wort are invariably recognized as cells, introducing error and variability to the measurements. Brewers with larger budgets often resort to using Coulter Counters (e.g. Coulter Z2) to precisely measure their yeast counts. Those systems have long been established as the "gold standard" in particle counts and sizing, providing highly-accurate and repeatible counts through the electrical measurement of the exact size (and concentrations) of tens of thousands of cells in a matter of seconds. The accuracy of the sizing information, in particular, allows for tracking of the yeast sizes throughout the brewing life cycle, a factor with demonstrated importance in the brewing process.[6] However, the main challenge with Coulter Counters had been the absence of viability information on the sample and, until the relatively recent introduction of the Orflo Moxi Z system, the high cost. Finally, at the highest end of the data quality spectrum, flow cytometers are beginning to emerge as a new gold standard in yeast counting and viability for control of brewing processes.[5] However, historically, the extraordinarily high costs and complexity of these systems have made them a tool for only the global brewers or academics.

Esterase Activity as a Measure of Yeast Health/Stress

The viability methods described above are all based on the simple principle of cell membrane exclusion. Briefly, as cells die, ruptures/holes form in the membrane that allow for the entry of larger molecules that ordinarily would be blocked. In this manner, viability dyes such as methylene blue and propidium iodide (PI) are excluded from the interior of "live" cells and allowed to enter "dead" cells. The result is that the dead cells accumulate the dyes and are either shaded with the dye's color (methylene blue) or brightly fluoresce (PI). This is the approach traditional used by brewers to assess the "viability" state of yeast. However, the exclusion of the dye does not reflect the metabolic (activity) state of the cell which is crucial to understanding whether the cell is actively converting sugar to alchohol or if it is stressed/dormant. And, the cell "viability" (as defined by permeability) is an end-stage marker, not providing brewers the opportunity to intervene in adjusting the environment to prevent (often wide scale) cell loss. As a supplement to membrane integrity, one common measurement of cell metabolism (often referred to as "vitality") is to use molecules (e.g. 5-carboxyfluorescein diacetate, acetoxymethyl ester or CFDA-AM) that load into all cells and are converted into fluorescent dyes by the action of esterase enzymes, an enzyme that has been found in almost all cells. In this way, the activity of a cell can be quantitatively monitored with higher cellular fluorescence reflecting higher cell metabolisms. A drop in esterase activity can serve as a potential signal to brewers to adjust operating conditions. And, beyond that, esterase activity also holds particular signifcance in brewing beyond just metabolic activity. Specifically, esterase has been shown to be important in helping to break down the esters, that can negatively impact taste, in high gravity brewing.[7,8] While microscopic approaches can be applied to esterase visualization, for proper quantitification of the fluorecence and statistical analysis, flow cytometry is the required technique for this type of analysis.

Orflo's Moxi GO II - Combined Flow Cytometer and Coulter-Principle Counter

Orflo's newest, "Next Generation Flow Cytometer", the Moxi GO II (Figure 1), is ideally and uniquely suited to enabling yeast monitoring in brewing. The Moxi GO II is the only system that combines the precision of Coulter Principle based cell sizing and counts with the flexibility of flow cytometry for viability and more advanced brewing analysis. The system combines the Coulter Principle, the recognized gold standard for precise cell sizing and counts, with simultaneous fluorescent measurements using a 488nm laser, coupled with two PMT detection channels, one filtered to detect at 525/45nm and the other at 561nm/LP. This fluorescence configuration is ideal for many of the most common fluorophores including propidium iodide (PI for viability), CFDA-AM (esterase activity), and tetramethylrhodamine (TMRE, mitochondrial potential). The Moxi GO II utilizes a disposable flow-cell architecture, does not require warm-up, runs test in under 10 seconds, and

does not require cleaning/shutdown procedures. Its low cost and very small footprint make it easy for brewers to acquire one for their brewery and place it conveniently near the tanks for instant feedback. In this way, the system can allow brewers to very easily track count, viability, and advanced health data over time. Furthermore, the touchscreen interface and OS were designed to be highly intuitive for users with no required flow cytometry experience. In this app note, we present data for the Moxi GO II system as it was applied to measuring the viability, esterase activity, and mitochondrial potential for an active brew at a local San Diego craft brewery.

Example Data – Results and Discussion

The general count and sizing performance of the technology underlying the Moxi GO II (compared to higher end Coulter Counters[9,10] as well as viability performance versus an imaging system and more expensive flow cytometer[11]) had been established in prior publications. In the data that follows, we focus on the specific application of the Moxi GO II to analyzing yeast samples from an actual brewing environment. Figure 2 shows example counts and viability (PI-based membrane permeability) output for San Diego Super Yeast (WLP090, White Labs) pulled from a local craft brewery bright tank. Figure 2a. shows the representative Moxi GO II viability output with a scatter plot display of PI fluorescence versus Coulter Principle cell sizing. In the course of a ~10 second test, the system clearly displays the sample viability (Figure 2a, yellow box, 94.0%) as well as the counts and precise sizing information (black boxes below the scatter plot). To highlight the capability of the system to discriminate live versus dead cells, half of the sample was intentionally heat-killed and processed by the system. While the live and heat-killed samples were run separately, Figure 2b and 2c highlight the capability of the user to use the system to easily overlay/compare two saved test results. Specifically, Figure 2a shows the user-generated system screenshots of the overlay of the traditional scatter plot output (PI fluorescence vs. Coulter Sizing) for the two samples: Healthy (gray dots) and heat-killed (colored dots) yeast. This overlay format clearly shows the dramatic increase in fluorescence of the heat-killed (dead, colored scatter plot) yeast sample as the PI enters the cell, binds the DNA, and increases fluorescence (~40x increase) – clearly differentiating live from dead cells. That increase in fluorescence is also readily visualized in Figure 2b that provides the test compare/overlay for the PI fluorescence histograms (gray curve =

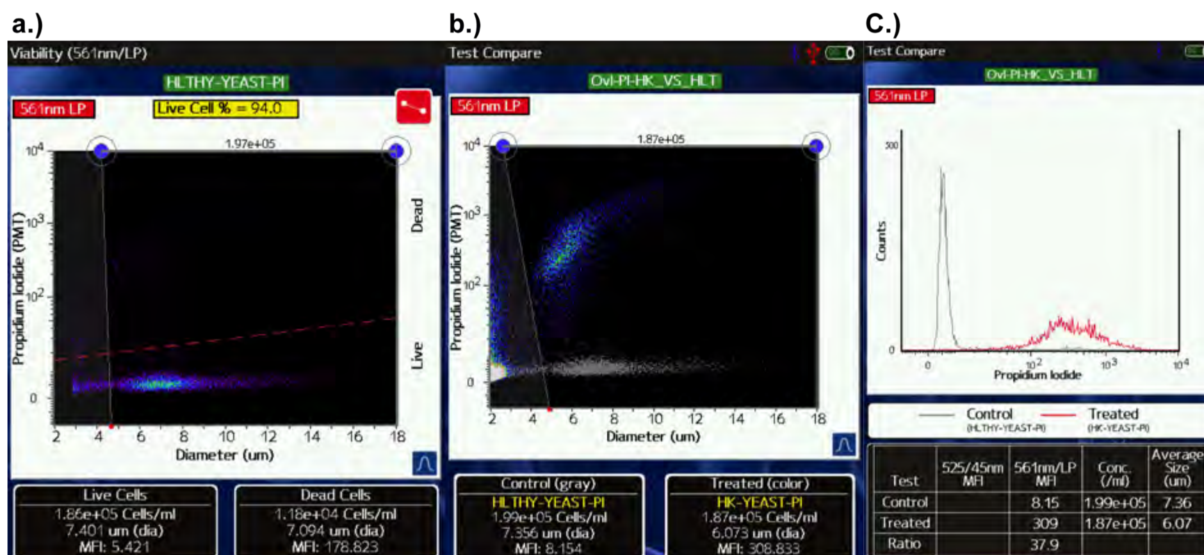


Figure 2 – *a.)* Representative Moxi GO II viability (PI) scatter plot (PI fluorescence vs. Coulter sizing) output for a live/healthy bright tank yeast sample. The 94.0% (yellow box) would be a typical, high, viability for such a sample. *b.)* The system is capable of generating comparison/overlays of Moxi GO II Viability (PI) data for two tests. Here a healthy bright tank yeast (gray scatter plot) sample is overlaid with a heat-killed (colored scatter plot) sample, showing the clear shift in PI fluorescence. *c.)* This data can also be displayed as PI fluorescence histograms with live/healthy yeast (gray curve) and heat-killed yeast (red curve).

healthy yeast, red curve = heat-killed yeast). In this way, not only does the system allow for the quick (10 second measurement) of sample viability information, it also lets the brewer rapidly compare that data to earlier time points to provide clear visualization of the sample viability progression.

The same yeast samples were incubated with CFDA-AM to measure the esterase activities (metabolic activity/cell health) of the yeast samples. CFDA-AM is a cell-permeant molecule (passes readily inside the cell) that is non-fluorescent in its intact state. However, the natural activity of the esterase enzyme that is present in virtually all healthy/active cells, will cleave the acetoxymethyl ester (AM) moiety from the molecule allowing the CFDA molecule to fluoresce when stimulated with the 488nm light (Moxi GO II laser). As CFDA is also charged, it is retained within the healthy cells (intact cell membranes) causing whole cell fluorescence that is easily quantified on the Moxi GO. Figure 3a shows an example of the CFDA fluorescence vs. Coulter Sizing scatter plot for a healthy yeast bright tank sample. As with the viability assay, the high percentage of fluorescent cells (96.6%, Figure 3a, yellow box) would be expected for a healthy yeast samples. However, unlike the PI-based viability approach, the median fluorescence intensity (MFI, Figure 3a, black box to lower right of the scatter plot) of the CFDA would be expected to shift in a manner proportional to the health of the cell. In this manner, the relative value of the CFDA fluorescence versus known-healthy and/or known-dead samples can provide valuable, relative, information on the potential decline of a yeast sample, likely before the cells stained "dead" with PI. An example overlay for the heat-killed (colored scatter plot) vs. healthy yeast (gray scatter plots) is shown in Figure 3b and Figure 3c provides the overlaid CFDA fluorescence histograms for the two samples (gray curve = healthy yeast, red curve = heat-killed yeast). In Figure 2c, note the CFDA MFI decreases from 1010mV to just 23.3mV, a change of 43x. In this sample, those values would define the dynamic range of possible activity states between a clearly-killed yeast sample and a 100% healthy sample, providing a powerful tool to quantify the intermediates in the activity of yeast cells in the tank.

In addition to these core assay capabilities, one of the powerful features of the Moxi GO II is that it is

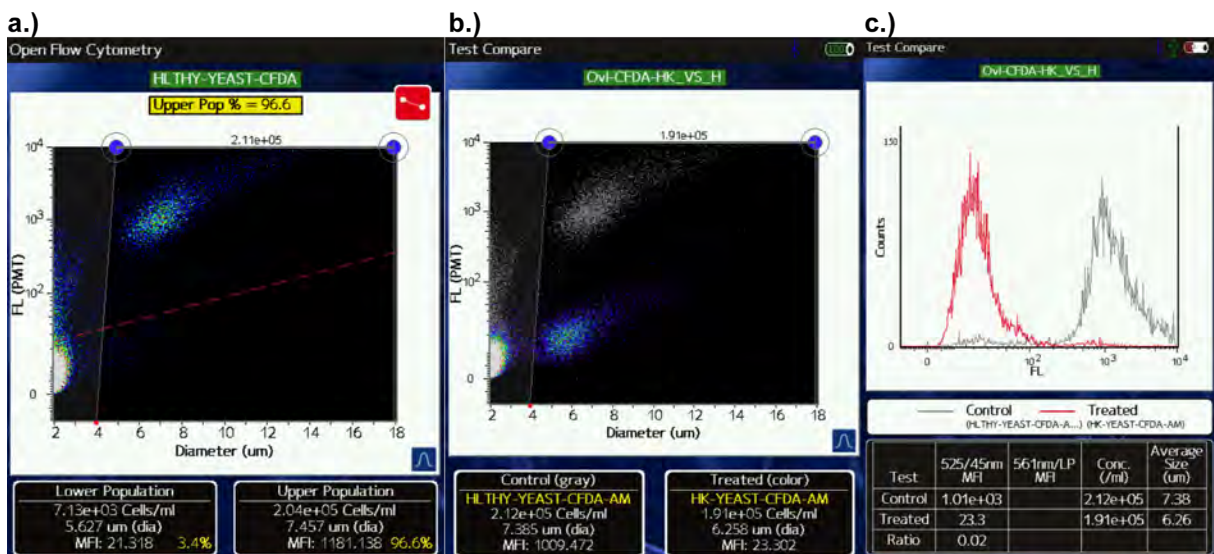
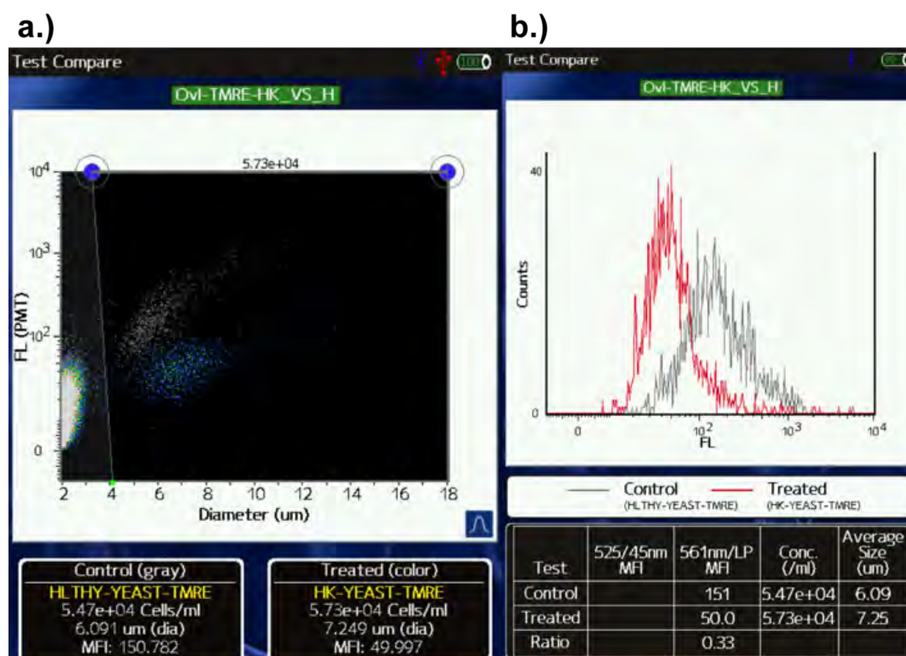


Figure 3 - *a.)* Representative Moxi GO II (CFDA-AM) scatter plot (CFDA fluorescence vs. Coulter sizing) output for a live/healthy bright tank yeast sample. CFDA-AM provides a measure of cell metabolic activity by reflecting the activity of the constitutive cell enzyme, esterase. So, the 96.6% (yellow box) level is typical for a healthy tank sample. *b.)* The system is capable of generating comparison/overlays of Moxi GO II Viability (CFDA) data for two tests. Here a healthy bright tank yeast (gray scatter plot) sample is overlaid with a heat-killed (colored scatter plot) sample, showing the clear decrease in CFDA fluorescence (esterase activity) for the heat-killed sample *c.)* This data can also be displayed as CFDA fluorescence histograms with live/healthy yeast (gray curve) and heat-killed yeast (red curve).

a true, open-platform flow cytometer. As such, brewers can use it to explore other fluorescent approaches to categorizing their yeast state. As new research is being continuously released regarding the state of yeast in brewing samples, it is likely that new flow cytometry analysis approaches, with the potential to revolutionize brewing, will also be identified. One example of an area that researchers are investigating is the relative contribution of yeast mitochondria to the brewing process and outcome. While it has long been assumed that mitochondrial activity is negligible in the anaerobic brewing environment, evidence suggests they may still serve a critical role in brewing.[12] As the mitochondrial potential has been linked to the level of flavor-contributing fatty acids in sake fermentation[13], we applied the Moxi GO II to the measurement of the mitochondrial potential in the yeast samples in this study as a demonstration of the flexibility of the system. Specifically, to measure mitochondrial potential we applied a dye, tetramethylrhodamine (TMRE) that is known to permeate cells and accumulate in mitochondria in proportion the level of charge (transmembrane potential) across the mitochondrial membrane. In Figure 4, we present the results of this analysis. Figure 4a shows the TMRE fluorescence (Mitochondria potential) versus Coulter Sizing scatter plot overlays for the healthy (gray scatter plot) yeast sample versus the heat-killed (colored scatter plot). Figure 4b represents the TMRE fluorescence-only histograms for the two samples (gray curve = healthy yeast, red curve = heat-killed yeast). In this case, an expected decrease is observed for the dead cells. While the mitochondrial potential for yeast in brewing would not likely provide value in a live/dead assay or its traditional use towards the measure of aerobic respiration, it might eventually serve as a metric in the isolation of certain undesirable flavors in the final output. Data such as this simply highlights the potential versatility and impact of the Moxi GO II in enabling more advanced brewing analysis in the future, beyond the core Moxi GO II assays of counts, viability, and metabolic activity.

Summary

In this application note, we describe the application of the Moxi GO II towards monitoring of yeast



Test	525/45nm MFI	561nm/LP MFI	Conc. (/ml)	Average Size (um)
Control		151	5.47e+04	6.09
Treated		50.0	5.73e+04	7.25
Ratio		0.33		

Figure 4 – Comparison/Overlays of Moxi GO II Mitochondrial potential (TMRE) data for a healthy bright tank yeast (gray scatter plot, gray histogram) sample vs. a heat-killed (colored scatter plot, red histogram) yeast sample displayed as *a.)* Scatter/dot plot of TMRE fluorescence vs. Coulter-Based Cell Size and *b.)* TMRE Fluorescence histogram. TMRE provides a measure of mitochondrial potential as it uptaken into mitochondria in proportion to the mitochondria charge.

in brewing. Data is presented to highlight the capabilities of the instrument to support the core requirement of highly accurate yeast counts and viability as well as the versatility of the instrument in measuring other parameters such as esterase activity and mitochondrial potential. One of the most powerful features of the Moxi GO II instrument is the ease-of-use and versatility in collection of data. With an ability to run tests without the need for system warm-up, maintenance, or shutdown procedures, the Moxi GO II is ideally suited for monitoring the time course of yeast health throughout the brewing process. This is further facilitated by the Moxi GO II's small footprint which enables brewers place the Moxi GO II right by the brewing tanks for more immediate and frequent flow analysis of their systems over (potentially) long periods of time. Finally the Moxi GO II touchscreen GUI is designed to make even complex flow analysis accessible to all users, regardless of their flow expertise. These features, combined with the system's low price point, should establish the Moxi GO II as a staple in brewery that is serious about better understanding and controlling the brewing process.

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Methods

Initial Cell Concentration Adjustments

Cells were sampled directly from a bright tank sample containing and unspecified batch of beer using San Diego Super Yeast (White Labs, #WLP090). The Sample was diluted 1000x by serial dilution. First, 100µL of tank sample was added into ~10mL of Orflo Diluent (Orflo Technologies, Cat #MXA006) for a 100x dilution. The 1000x dilution was then realized by making an additional 10x dilution by taking 1ml of the 100x sample and diluting it into 9ml of Orflo Diluent. Diluted concentrations were verified on the Moxi GO II system.

Heat Killed Yeast Cell Controls

To achieve a dead-cell control for the various assays, a heat-killed yeast sample was generated. Water was brought to a boil in an electric kettle and transferred to a beaker and allowed to cool for 1 min. A 5ml sample of the diluted yeast was aliquoted to a 15ml centrifuge tube and placed in the hot water beaker for 10 minutes.

Cell Assays

All yeast cell assays followed the corresponding Orflo Protocols (included below). The PI viability assay used the "Orflo Moxi GO II/Moxi Flow Viability (PI) Staining Protocol" using the Orflo Flow Reagent optional step. The esterase-activity was prepared using the "Moxi GO II- Cell Health/Vitality/Esterase Activity with CFDA-AM" protocol, following the steps for CFDA-AM inclusion. The final CFDA-AM incubation concentration was 5μM, the incubation time was 30minutes, and the optional Orflo Flow Reagent was added. The mitochondrial potential assay was implemented according to the "Moxi GO II – Mitochondrial Membrane Potential – TMRE Staining" protocol. The final TMRE concentration was 300nM, the incubation time was 20 minutes, and the optional Orflo Flow reagent was added.

Moxi GO Tests and Data Output

All tests were performed on a Moxi GO 488nm (Orflo Cat#MXG002) system. The viability assays, and initial cell concentration determination, were run using the "Viability Assay" functionality built into the firmware using the 561nm/LP filter (Orflo Cat#MOG002). The CFDA-AM assay was run using the "Open Flow Cytometry" functionality, "Medium" fluorescent gain, and the 525/45nm filter (Orflo Cat#MXG001). *Note: The Moxi GO used in this study is simply the 1 PMT/channel version of the Moxi GO II system. Other than the PMT configuration, the system has the same architecture as the Moxi GO II so performance would be equivalent*. The TMRE assay was run using the "Open Flow Cytometry" functionality, "Medium" fluorescent gain, and the 561nm/LP filter (Orflo Cat#MXG002). Data comparisons/overlays were all performed on-unit using the built-in system functionality for comparing saved tests. Screenshots were all generated directly from the unit using the built-in "Print" functionality to export data screenshots in bitmap (BMP) format. Final image cropping and arrangement was performed using Photoshop (Adobe) and Illustrator (Adobe).

Moxi GO II/Moxi Flow – Viability (PI) Staining Protocol

Overview:

This viability protocol is based on the simple principle of cell membrane exclusion. Briefly, as cells die, ruptures/holes form in the membrane that allow for the entry of larger molecules that ordinarily would be blocked. In this manner, viability dyes such such as propidium iodide (PI) are excluded from the interior of "live" cells and allowed to enter "dead" cells. The result is that the dead cells accumulate the dyes and brightly fluoresce (PI). PI is particularly attactive as it exhibits a ~40x increase in fluorescence upon binding with DNA (after it enters the cell) resulting a large signal increase, relative to the shading level change associated with Trypan Blue.

Reagents required:

- Dilution Buffer (e.g. PBS)
- Propidium Iodide (PI) stock solution - 1mg/ml (e.g., ThermoFisher Cat#P3566)
- *Optional/Recommended:* Orflo Flow Reagent (Orflo Cat #MXA080)

Viability (PI) Staining

1. Dilute cells to 1 - 5 x 10^5 cells/ml (recommended range) using Dilution Buffer
   Note: As a rule of thumb, for samples with unknown concentrations, start with a 10x dilution.
2. Add 2μL of (1mg/ml) PI stock solution for every 1mL of cell solution (final PI concentration of 2μg/mL).
3. Incubate for 5 minutes in the dark at room temperature (25°C).
4. *Optional/Recommended:* Add 20μL of Orflo Flow Reagent per ml of cell sample.
5. Analyze with the Moxi Flow or Moxi GO II using the "*Viability Count (PI)*" app within 10 minutes.

Moxi GO II - Cell Health/Vitality/Esterase Activity with CFDA-AM

Overview:

Traditionally, cell "viability" measurements rely on the simple principle of cell membrane exclusion (i.e. larger molecules, such as dyes, are excluded from the interior of cells). However, the simple exclusion of a dye does not necessarily reflect the metabolic state of the cell which many researchers regards as a more critical metric of cell viability or "vitality." Towards this end, several dyes (e.g. 5-CFDA) are available that are designed to measure the esterase enzyme activity, a family of enzymes believed to be common to all cells. To measure this activity, the dyes are conjugated to acetoxymethyl ester moieties (e.g. 5-CFDA-AM) that serve to simultaneously assist in the membrane permeability of the dyes as well as quench the dyes fluorescence. Once loaded, the constitutive activity of the esterase enzyme cleaves the AM functionality of the molecule causing it to fluoresce and slowing the rate of effusion from the cell. The result is a measureable cellular fluorescence that can be quantified and correlated to the metabolic state of cells. This protocol describes the implementation of this "Cell Health" or "Vitality" assay on the Orflo Moxi GO II system.

Instrument/Cassettes:

- Orflo Moxi GO II Next Generation Flow Cytometer (Orflo Cat #MXG102)
- Compatible Orflo Cassette (any of the following would work):
  - Type CST (Orflo Cat# MXC040)
  - MF-S (Orflo Cat# MXC020)
  - MF-S+ (MXC030)

Reagents:

- 5-CFDA, AM (e.g. ThermoFisher, Cat# C1354)
- Phosphate Buffered Saline (PBS, e.g. Thermo 10010023)
- DMSO (any brand, e.g. Sigma #D8418)
- Propidium Iodide (PI) staining solution (e.g. Thermo P3566 (1mg/ml PI))
- DMSO (any brand, e.g. Sigma #D8418)
- *Optional/Recommended: Orflo Flow Reagent (Orflo Cat #MXA080)*

CFDA-AM Dye Stock Solution Preparation

- Create a 1mM stock solution of the AM dye:
  - CFDA-AM
    - Add 9.39mL DMSO to 5mg CFDA-AM stock
    - Mix to ensure it fully dissolves and aliquot (e.g. 200µL vials) to minimize the potential for hydrolysis with repeated opening/closing and freeze thaw cycles.

Protocol:

1. Dilute cells with PBS to get sample into the optimal concentration range for the Moxi Flow. Final target concentration: ~1e5 – 5e5 cells/mL.
2. For each sample to be stained, add 500µL of diluted cells into a microcentrifuge tube. Note: It is useful to prepare a negative control by heat-killing a sample (60-70°C, 10min). For compensation, prepare three tubes:
   a. PI only sample with the heat killed cells
   b. CFDA-AM only sample with the Healthy/Live (control) cells
   c. CFDA-AM and PI Sample for target cells

3. Add 2.5µL of 1mM CFDA-AM stock for a final concentration of 5µM CFDA-AM. *Please note that, for different yeast cell lines, it may be necessary to perform a titration of the CFDA-AM concentration to determine the optimal staining concentration to prevent railing of the system (too bright) or to achieve sufficient signal (to dim).*
4. Add 1µL of 1mg/ml PI to the appropriate tubes (final concentration 2µg/ml, 3µM)
5. Vortex gently, Incubate samples at 37°C for 15min in the dark. Longer incubation times can be applied for increased CFDA-AM signals. *Note: If longer times are used, we would recommend adding the PI 5-10minutes prior to the end of the CFDA-AM incubation.*
6. *Optional/Recommend:* Add 10µL Orflo Flow Reagent to each sample and inversion mix.
7. Run on Moxi GO II using the "*Cell Health (Calcein-AM & PI)*" app immediately:
    a. Adjust size gates to define the cell population.
    b. Touch "X/Y" to select a PMT vs PMT display of the CFDA-AM (PMT1) vs. PI (PMT2) fluorescence.
    c. Adjust for spillover as appropriate using the "Comp" button functionality.

Moxi GO II - Mitochondrial Membrane Potential TMRE Staining

Overview:

Tetramethylrhodamine, ethyl ester (TMRE) is a cell-permeant fluorescent dye that, due to it's cationic nature, is readily sequestered by active mitochondria. TMRE has a peak excitation of 549nm but can be excited by either the 488nm or 532nm laser. The peak emission is 574nm, requiring the use of the 561nm/LP channel on the Moxi GO II instrument.

Instrument/Cassettes:

- Orflo Moxi GO II Next Generation Flow Cytometer (Orflo Cat #MXG102)
- Compatible Orflo Cassette (any of the following would work):
  - Type CST (Orflo Cat# MXC040)
  - MF-S (Orflo Cat# MXC020)
  - MF-S+ (MXC030)
  -

Reagents/Components:

- Tetramethylrhodamine ethyl ester perchlorate (e.g. Sigma Cat #87917)
- Cell Staining Buffer (BioLegend cat # 420201 or PBS with 0.5% BSA, 0.1% Azide). *Note: Stock PBS (any formulation, e.g. Gibco, Cat #10010023) can be used but might have slightly lower signal-to-noise ratios (higher background) for the assay.*
- DMSO (any brand, e.g. Sigma #D8418)
- *Optional/Recommended*: Orflo Flow Reagent (Orflo Cat #MXA080)

Mitochondria - TMRE Labeling Protocol

1. Make initial stock solutions of TMRE by sequential dilution of TMRE (Sigma Cat #87917) as follows:
   a. 10mM stock TMRE: 25mg dissolved TMRE in 4.85ml DMSO
   b. 10µM stock TMRE: 10µL of 10mM TMRE stock in 990µL DSMO
2. Dilute cells to a concentration of ~2e5-3e5 cells/ml with Cell Staining Buffer. *Notes:*
   a. *Cells can be labeled directly in in culture media if they are already at the correct concentration (or are under-concentration).*
   b. *Cells need to be in a single cell suspension for testing. Detachment with Accutase/Accumax is recommended with pipette trituration to break apart clusters*
3. Aliquot 500µL of cell suspensions (it is useful to generate an FCCP-treated (i.e. 100µM), Sigma Cat #C2920, sample as a negative control) into separate polypropylene micro-centrifuge tubes (Santa Cruz, Cat #sc-200271) – *Note: do not use polystyrene (PS) as TMRE can bind significantly to PS.*
4. Add 2.5µL of 10µM TMRE stock solution to each vial to achieve a 50nM final TMRE concentration and gently vortex to disperse. *NOTE: At high overly high concentrations, TMRE has a quenching effect. Consequently, optimal TMRE concentration is dependent on cell type and sample prep and can vary from 20nM –200nM. Initial titration tests should be performed for optimal concentrations. As an initial guess, start with 50nM TMRE.*
5. Incubate TMRE/Cell media at 37°C for 15-30 minutes in the dark.
6. Post-incubation, incubate cells for an additional 15min (RT/Dark).
7. *Optional/Recommended: Add 20µL Orflo Flow Reagent per ml of cells and inversion mix sample.*

8. Following dilution, immediately run the samples on the Moxi GO II system using the “Open Flow Cytometry” assay with PMT2 (561nm/LP) selected and the “Default” gain setting.