Flow Cytometry — A Primer for Modern Analytical Technology to Assess Yeast Culture Quality for Fermentation

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Flow cytometry is an analytical technology that allows users to interrogate single cells at high speeds to provide a detailed data-rich analysis of complex cell populations. Flow cytometry can offer an effective means to quickly evaluate yeast culture integrity using a small sample to assess the complexity and underlying heterogeneity in a culture. It can assess cell viability, purity and complexity, with respect to potential contaminating species, of the culture providing a higher level of confidence in the population to be used for fermentation. This is an important problem to address in breweries where multiple domestic yeast strains are employed or where various wild strains of *Saccharomyces cerevisiae* as well as wild or cultured *Brettanomyces* species are used for fermentation. Such analysis can enhance confidence and reduce costs associated with new yeast culture purchase in commercial fermentations and promote a deeper understanding of the complexities of mixed fermentations. Lastly, flow cytometry can add new levels of analysis associated with fluorescent labeling of cell DNA content and selected cell proteins.

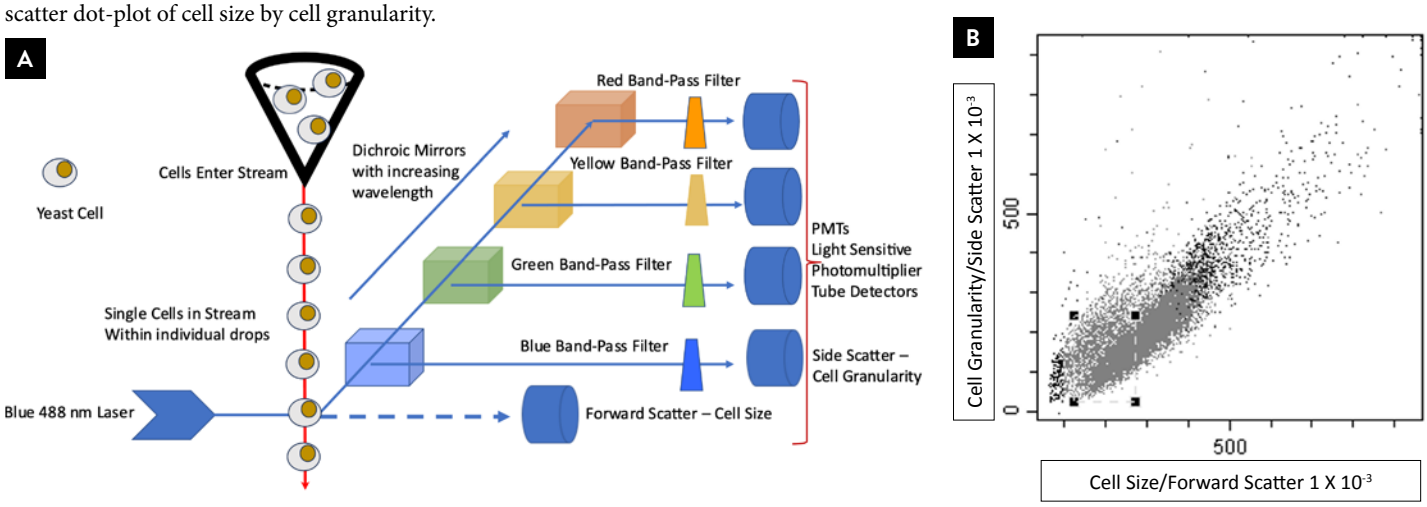
INTRODUCTION

Flow cytometry is an analytical technology that allows users to interrogate single cells one at a time at very high speeds to provide a detailed data-rich analysis of complex cell populations. Flow cytometry has been a staple cell analytical tool in basic cell biology and immunology, as well as clinical applications of these disciplines, for decades. Beginning with the first laboratory-built instrument by Göhde, University of Münster in 1968, the first rudimentary commercial instrument by Partec (Göttingen) quickly followed in 1969 [1]. By 1978 flow cytometry had been coined as a name for this technology and soon after several commercial instruments were introduced. They were limited in analytical ability, used powerful and dangerous water-cooled lasers, and were challenging to successfully operate. This has all changed in the present with the development of higher-powered solid-state lasers and high efficiency digital optical arrays [2]. Currently, more than a dozen companies produce flow cytometers from very high-end biomedical instruments, with 6 lasers and 21 fluorescent channels costing up to half a million dollars (Beckman Coulter, BD Instruments), to much more affordable entry-level benchtop instruments (around $20,000) that have one or two smaller diode lasers and can detect 2-4 fluorescent channels (BD Instruments, Millipore, Miltenyi). Most cytometers use digital photomultiplier tubes (PMTs) to detect light emissions, but some more advanced instruments can incorporate advanced avalanche photodiodes or even spectral flow cytometry. Another advance has been the ability of these newer instruments to detect smaller particles/cells down to as small as 100 nm in the case of higher end instruments. Because *Saccharomyces* run in the 4 µm range (40 times this minimum) they are easily detected in a modern flow cytometer. All of this makes flow cytometry particularly ready to have an important impact on fermentation analysis of yeast quality, purity, and viability.

WHY FLOW CYTOMETRY FOR YEAST USED TO FERMENT BEER AND SPIRITS?

Brewers are concerned over the quality of their yeast pitches, whether for brewing beer or distillation, and often replace in-house cultures, at considerable

FIGURE 1 *Diagrammatic View of the Optical and Cell Analysis Array in a Flow Cytometer.* **A:** Schematic representation of the fluidic and optical arrays of a typical single blue laser flow cytometer that detects forward scatter (cell size), side scatter (cell granularity), and three fluorescent color channels (green, yellow, and red optical emissions). Each cell enters the high-pressure sheath fluid stream in single file (red arrow) until they are interrogated by the blue 488 nm laser line. Each cell creates a 2-dimensional shadow used to calculate cell size directly in line with the laser beam (dotted blue arrow) while light emitted at 90° to the laser beam is collected and separated into 4 different color channels for detection by PMTs – photomultiplier tubes that detect incident light. Dichroic mirrors filter the blue light allowing only light beyond a certain limit to pass but reflects blue wavelengths and below toward the PMT where the light is further restricted by a band-pass filter that only allows passage of blue light to excite the PMT. For each cell data is collected by all detectors and collated in a data packet associated with just that cell. **B:** The lower panel shows a standard forward by side scatter dot-plot of cell size by cell granularity.



expense (this can be hundreds of dollars for each new yeast block), if they are insecure regarding the integrity of the culture and the risk of infection. All brewing is, by necessity, only sanitary and not completely sterile so the chances of picking up contaminating strains approaches inevitable through manipulation during each fermentation cycle. However, many breweries with excellent aseptic technique do successfully serially passage yeast cells for many generations/brews, exceeding 20 times in some cases, without noticeable contamination problems. All brewers, however, carefully monitor the quality and integrity of their yeast.

Flow cytometry can offer an effective means to evaluate yeast culture integrity using only a small sample (a few microliters to 1 mL) to quickly assess the complexity and including any underlying heterogeneity in a sampled culture due to viability issues or contaminating species. It can assess culture quality providing a higher level of confidence in the population about to be pitched into a fermentation [3]. This is an important problem to address in breweries where multiple yeast strains are employed including ale (*Saccharomyces cerevisiae*), lager (*Saccharomyces pastorianus*), and various wild strains, including wild isolates of *Saccharomyces cerevisiae*, and any of several wild and cultured *Brettanomyces* species [4,5]. Such analysis can enhance confidence and reduce costs associated with unnecessary new yeast culture purchases in commercial fermentations. Analysis can usually be obtained from flow cytometry labs for very reasonable cost.

HOW FLOW CYTOMETRY WORKS AND THE TECHNICAL ADVANTAGE

Flow cytometry is the process of analyzing the physical and biochemical characteristics – the phenotype – of biological cells. Because it analyzes each cell individually it is capable of distinguishing different cell types with different phenotypes and applying this to very complex cell populations. This is much more powerful than measuring average characteristics for a quantity of liquid culture and is capable of rapidly (within minutes) analyzing vast quantities of cells compared to plating on agar.

Flow cytometers work by drawing up cell suspensions from a sample tube in extremely narrow streams such that individual cells pass through the laser paths one at a time. This requires that yeast be diluted to approximately $1x10^6$ – $1x10^7$ cells/mL. Because yeast often grow to cell densities 1000x this density, dilution is necessary but simple to accomplish by dilution in water or PBS (phosphate-buffered saline at 0.9% $NaCl_2$, 10 µL per 10 mL or 1 drop - approximately 50 µL - of liquid culture in 50 mL water diluted to less than 1x107 cells/mL). A schematic representation of the fluidic and optical arrays of a typical single blue laser flow cytometer that detects forward scatter (cell size), side scatter (cell granularity) and three fluorescent color channels (green, yellow, and red optical emissions) is shown (**Figure 1A**). Initially, each cell enters the high-pressure sheath fluid stream by being introduced into that stream at a pressure slightly higher than the sheath pressure (red arrow). Cells flow down, within the

stream in air, in a laminar flow until they are interrogated by the blue 488 nm laser. Each cell creates a 2-dimensional shadow that is used to calculate cell size directly in line with the laser beam (dashed blue arrow). Light emitted at 90° to the laser beam is also collected and separated into 4 different color channels: green, yellow, red, and blue. Each different colored light emission is detected by a separate PMT – photomultiplier tube that detects incident light. To restrict each PMT to a narrow band of light, dichroic mirrors are added to filter the blue light allowing only light beyond a certain limit to pass. Thus, the first dichroic allows light above green wavelengths to pass but reflects blue wavelengths and below toward the PMT. Then the light is further restricted by passing through a band-pass filter that only allows passage of a very narrow band of blue light to excite the PMT. This ensures each PMT only sees a very narrow band of wavelengths to detect and quantify. Each color to be detected is reflected in a similar manner to a separate PMT. Each cell is interrogated by all detectors and any data is collected by the instrument computer and collated in a data packet that is associated with just that cell. The lower panel shows a standard forward by side scatter dot-plot of cell size by cell granularity that results from such analysis (**Figure 1B**). Each dot represents an individual cell, and this provides the power of flow cytometry. Unlike simple averages of whole populations, the population complexity, and thus the presence of small unhealthy cells or contaminating cells, can be easily identified.

Samples are loaded into the cytometer and flow through a channel or stream through which one or more lasers are aimed. By analyzing the optical output, the operator can determine the character or phenotype of the cells individually as they pass through the laser path. Each cell is interrogated as it passes through the laser path and multiple characteristics are analyzed and recorded including cell size, cell granularity, and lastly fluorescence in multiple spectral channels [6,7]. This last character will require the use of specific fluorescent labeling molecules – fluorochromes – to take advantage of it. Fluorochromes are organic molecules that absorb a specific wavelength of light (the excitation wavelength) and then re-emit that light while undergoing a red-shift to a longer wavelength (the emission wavelength). Fluorescent labels that specifically analyze DNA content, cell viability/integrity, and cell-specific surface proteins can be employed and each fluorochrome emits a characteristic wavelength/color of light that can be individually detected by the flow cytometer. The optical array on each instrument can thus analyze multiple color channels simultaneously promoting the assessment of many different fluorochromes and their associated targets in single cells. Such an approach allows detailed analysis of large populations of cells individually and then presents each parameter in a multidimensional plot to characterize the cells analyzed. At a minimum, forward scatter (cell size) and side scatter (cell granularity) can be assessed, and this can provide a real view into the complexity of the culture including quantification of any contaminating strains if they are sufficiently different from the intended brewing strain of yeast [2,8-10].

TECHNICAL REQUIREMENTS AND BASIC FORWARD BY SIDE SCATTER ANALYSIS

Once the user has access to a flow cytometer, individual samples from diluted cultures (usually $1x10^7$ cells/mL or lower – a 100-fold dilution of a stationary culture is usually adequate) can be quickly and efficiently analyzed within a few minutes. Such samples of live cells can be analyzed directly to assess both the cell size (forward scatter – a key characteristic of different species) and cell granularity (side scatter) of each cell. Granularity is a measure of how much of the laser light that enters the cell is refracted by surfaces and membranes into a path 90° to the incident path of the laser (**Figure 1**). Such basic analysis can reveal much regarding the yeast population including cell size and diversity of the cells present, including the presence of more than one microbial species, and thus if there are potential contaminants evident. Because analysis is on single cells, even small numbers of contaminants can be efficiently detected and quantified. All that is required is a small yeast sample composed of single cells (less than 1 mL) [11]. It is important to note that single cells are essential as the flow cytometer will regard doublets or larger as single particles. Most cytometers can discriminate such multiple cell complexes which can be eliminated from the analyzed data for single cells by creating an analysis gate and removing such doublets from the population analyzed.

EXCITATION AND EMISSION SPECTRA

More detailed analyses will require the use of some type of fluorochrome that binds DNA or binds to a cell surface protein. The former is the most commonly used and those that require ethanol fixation to penetrate the cell can also be used to assess viability in unfixed cells as they will only stain dead cells in a living population. Stains that can penetrate living cells without ethanol fixation can be used directly on live culture samples to be assayed and provide a direct measure of DNA content [12-15].

One of the most important issues to understand is the concept of excitation and emission spectral optima for each fluorescent molecule used to label cells. While forward

scatter (cell size) and side scatter (granularity) do not depend on wavelength of incident laser light all other fluorochromes used will have such optima. Each fluorescent molecule will absorb light with an optimal wavelength meaning light that is more distant from the optimal wavelength will be absorbed less efficiently. Thus, any resulting emissions from the fluorochrome will also be reduced with increasing distance from the optimal excitation wavelength. To generate maximal fluorescent emissions, it is necessary to excite fluorochromes with laser light as close to the optimum excitation wavelength as possible. This is why more advanced flow cytometers can include as many as six lasers to accommodate as many and as wide a range of fluorochromes as possible. Fortunately for brewers, the needs for yeast analysis are much more modest and so is the instrument cost.

FIGURE 3 *PI Staining of Fixed Cells Detecting Total DNA Content and Cell Cycle.* An example of cells labeled with propidium iodide – a DNA binding stain – following fixation with ethanol that removes the cell membrane allowing entry of the stain into the cell. The stain combines with the DNA by intercalation – slipping between the bases of DNA and then when excited with blue light will emit a bright orange/red light. The amount of DNA present is encoded as increased movement to the right on the X-axis. Thus, cells that are proliferating actively pass through each of the cell cycle phases from G1, S to G2/M where they divide and begin the cycle again. Analysis of such samples allows quantification of cells in each cell cycle phase as well as staining apoptotic cells with less than the G1 phase level of DNA per cell. Cell clusters are also identified and separated from single proliferating cells. Note that cells in G2/M phases have twice the amount of DNA as G1 phase cells once they pass through S phase where the DNA genome is duplicated.

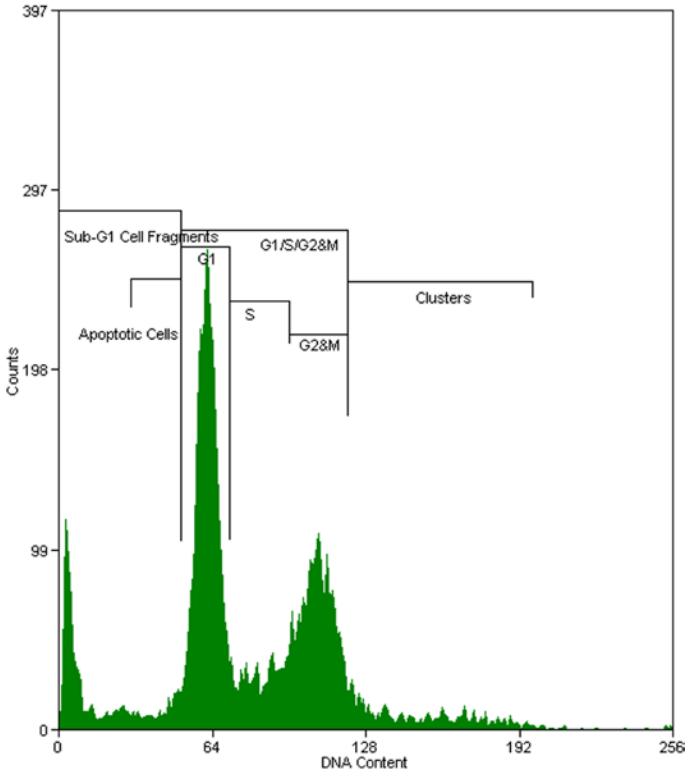
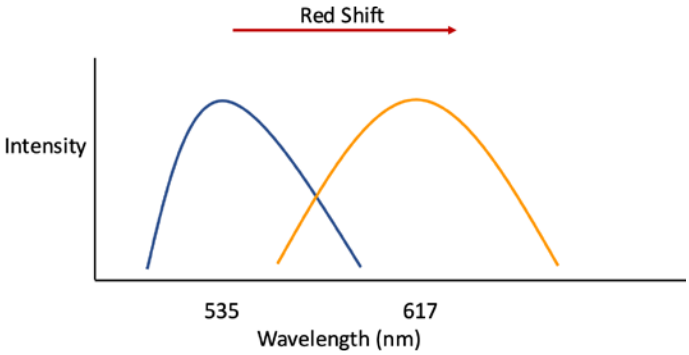


FIGURE 2 *Excitation and Emission Spectra for Propidium Iodide.* Each fluorochrome has a characteristic excitation and emission spectra. The two spectra are distinct, and emission is always at a wavelength that is longer than excitation – a red-shift. These specific characteristics allow selection of optical filters that can be used to precisely quantify the light emitted by that fluorochrome in the cell suspension independent of any other fluorochromes present. When a laser or filter set that is set to the precise optimum for that fluorochrome is not available, the flow cytometer can still detect the fluorochrome. However, as excitation wavelength moves off the peak for excitation there will be a proportional reduction in emission as less of the light energy is absorbed and so less light will be emitted.



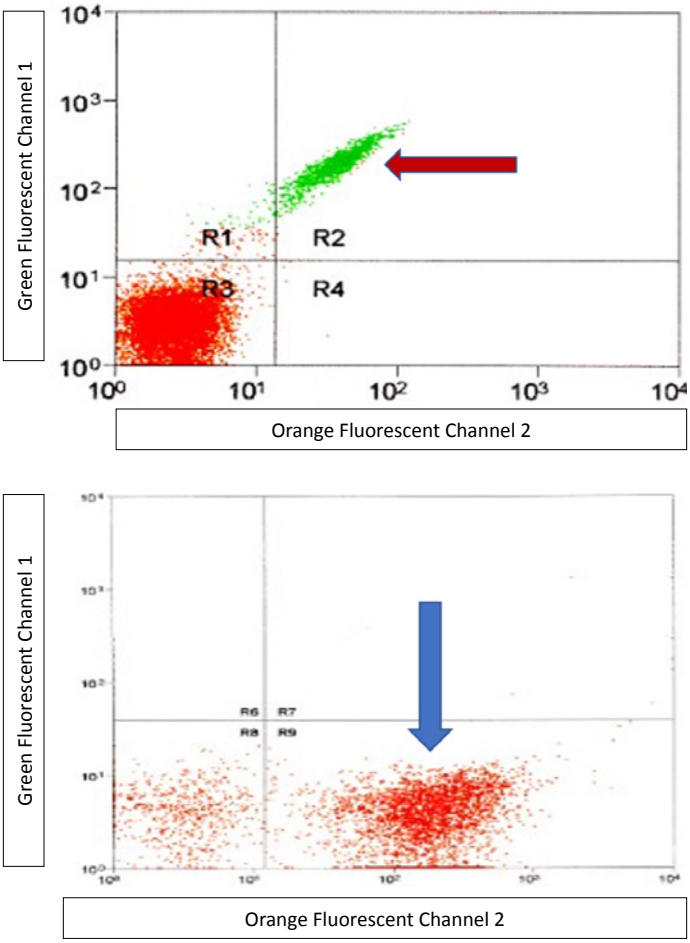
Most needs can be achieved using a small single blue diode laser, emitting light around 488 nm, which will excite a variety of fluorescent molecules useful in the analysis of yeast phenotype and viability [11].

When laser light excites a fluorochrome, even for a few microseconds, the light near the excitation optima is absorbed and then re-emitted undergoing the previously described red-shift. Or stated alternatively, a shift to a longer wavelength than the excitation wavelength – a shift toward the visibly red end of the spectrum. This characteristic emission is the one element that can be used to identify the fluorochrome emitting the light. The emission wavelength must be matched by appropriate optical detection arrays in the flow cytometer that are designed to filter the light such that only a narrow wavelength band, centered on the optimal (maximal) emission wavelength characteristic of the fluorochrome, is detected with maximum sensitivity (**Figures 1 and 2**). Such an array of dichroic and bandpass filters allows the precise parsing of emitted light into characteristic wavelength band-specific emissions that can selectively and precisely measure multiple wavelengths simultaneously. Color/wavelength indicates the fluorochrome detected, brightness of each cell (mean channel fluorescence for a population) which indicates the number of bound fluorochrome molecules per cell, and number of positively stained cells that indicates the relative number and abundance of cells expressing this marker in the population. This data is collected in addition to cell size and granularity data for each cell.

As each cell passes the laser and is illuminated, it absorbs the light and re-emits it at a wavelength that is characteristic of the fluorochrome used to label that cell. To provide a specific example, consider a cell population whose DNA is labeled with propidium iodide (PI) and illuminated with 488 nm blue light. The excitation optimum for PI is approximately 535 nm (close to the 488 nm light emitted by the laser) and the emission optimum is approximately 615 nm of orange/red light shifted toward the red part of the spectrum well above the incident blue light (**Figure 2**). For the example, this will result in a histogram depicting DNA content against cell frequency where higher DNA content per cell is found in cells as they occur more to the righthand end of the plot on the x-axis and this will result in detection of a classic cell cycle (**Figure 3**).

The complexity of this analysis can be greatly enhanced as instruments available can simultaneously analyze up to 24 different colored fluorochromes labeling one or more cell types in a population. Normally, however, such complexity is beyond the needs of yeast biologists and brewers, but the potential is there. If two colors are used in an analysis, then the operator must ensure that overlap in the emission spectra is minimized. If overlap occurs all is not lost as modern cytometers can employ advanced software to manage this and separate, or deconvolute, the two overlapping emissions using compensation applications within the cytometry analysis software. As an example, a cell population can be labeled with two fluorochromes emitting green and orange light. Both emissions will be visible in both spectral analysis windows as these colors share a yellow primary color base. Compensation is a software algorithm included in most cytometry software, that allows the users to move a single labeled cell population out of the double-labeled quadrant and back into the single-labeled quadrant in which it should reside (**Figure 4**). In the example, the green population is reduced in apparent fluorescence in the FL1 channel so that the MCF, or Mean Channel Fluorescence for the population, is now equal to unlabeled cells in the FL1 channel (fluorescence in quadrants R8 and R9 are equivalent in the FL1 channel). Once compensation is applied, the MCF value for cells with more than one fluorochrome label will be corrected such that cells with single labels can be identified correctly. This has the effect of pulling cells from a double-labeled region into a single labeled region thus removing the overlap in spectra and the ensuing analysis. Compensation is set up to precisely identify each labeled cell population, in a complex sample, and calibrated, before the analysis of the sample of interest, using cells labeled with only one of the fluorochromes, to be used, at a time. In this way, software can separate and still quantify the spectral emissions that overlap without losing any of the data (Kalusa, Beckman Coulter Inc, or FloJo, FlowJo Inc).

FIGURE 4 *2-Dimensional Dot-Plot of Two Fluorescent Channels Before and After Compensation.* A two-dimensional dot-plot of two fluorescent channels demonstrating compensation. Part of a cell population labeled with a single fluorochrome shows up as positive for two different fluorochromes (green population/red arrow) because the fluorochrome emissions bleed into the channel on the Y-axis. Compensation allows correction of this problem by lowering the MCF of the green population so that it is equal to the MCF of an unlabeled population (orange population bottom panel) on the Y-axis. Once applied, both populations are now negative for fluorochrome 1 (FL1) staining as compensation has been applied (blue arrow) and both populations are equal for MCF on the Y-axis.



DYES AND FLUOROCHROMES

The dyes and fluorochromes used to stain yeast cells vary in what kinds of cells they can permeate, the cellular structures and molecules they bind to, and the wavelengths of light where they emit maximum/optimal fluorescence. Several different types of dyes and fluorochromes are available.

FUN 1 — This fluorescent stain exhibits two-color fluorescence and can be used to indicate yeast cell viability (FUN 1 Cell Stain, Thermo Fisher Scientific). The stain is capable of passive movement across the yeast cell wall and plasma membrane. Generally, the dye first stains the

cytoplasm resulting in a scattered green fluorescence in the cell, but in living yeast, it is further processed and forms distinctive vacuolar assemblies which fluoresce bright red. The formation of the structures is coupled with a lessening of the green cytoplasmic fluorescence. This processing by the yeast cells requires that the plasma membrane is intact and that the cell exhibits metabolic activity, thus making it an exceptional marker of viability [16-18]. In contrast, the dead yeast cells demonstrate a yellow-green fluorescence with red structures lacking.

Propidium Iodide (PI) — This fluorescent stain binds to nucleic acids in the cell and fluoresces orange/red (Propidium Iodide, Thermo Fisher Scientific). PI will stain the DNA of fixed cells providing a classic cell cycle to ensure the population is growing. Living cells, with intact plasma membranes, are generally impermeable to the stain; however, there is some evidence that PI may be capable of staining stressed yeast cells with damaged, but repairable, plasma membranes [9,11,17-21,24-26,28,29]. As such, care should be taken in interpreting viability results when the strains used are chemically or environmentally stressed.

SYTOX — This group of dead cell stains, like PI, bind to DNA and cannot permeate the plasma membranes of living cells (5 mM SYTOX Green Nucleic Acid Stain in DMSO, Thermo Fisher Scientific) [8,12-15,21,24]. Depending on the SYTOX stain used, the cells will fluoresce blue, green, orange, or red thus allowing for flexibility in studies examining multiple parameters.

SYBR Green I — This stain strongly binds to double-stranded DNA and demonstrates high fluorescent yields compared to many other fluorochromes (Molecular Probes Thermo Fisher Scientific) [15,21,23,24]. A benefit of SYBR Green I is that it provides excellent high-resolution DNA measurements in cell cycle studies. As the name implies, cells stained with this dye fluoresce green.

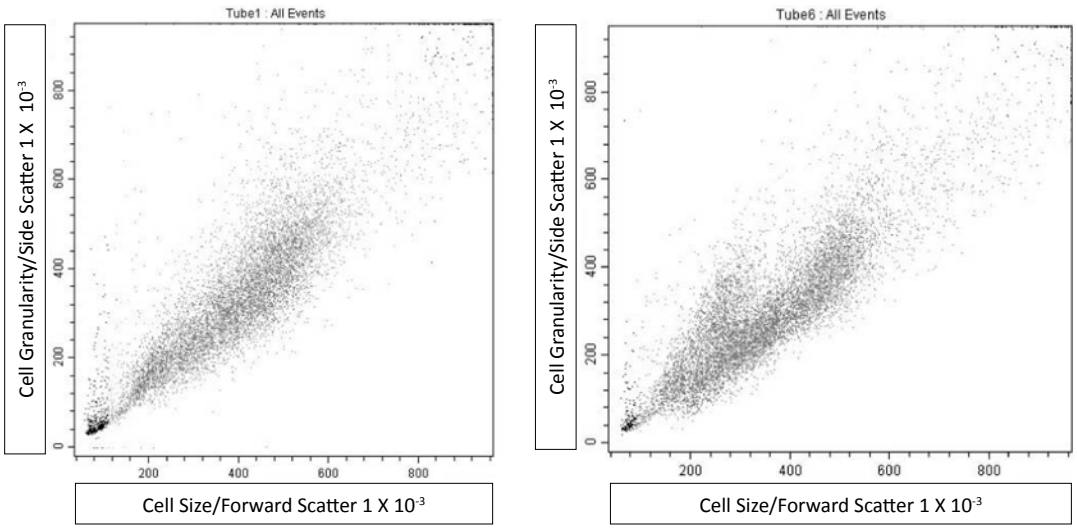
Vybrant DyeCycle Stains — Stains that can penetrate living cells without ethanol fixation, such as the Vybrant DyeCycle stains, can be used directly on eukaryotic cell cultures to be assayed and provide a direct measure of DNA content as stain binding and fluorescence is proportional to DNA content. This can also provide an assessment of cell cycle phase distributions in living cultures [22]. While there is extensive literature on staining animal cells, there is little data on yeast staining at present.

With stains that dye DNA, such as PI, SYTOX, and SYBR Green I, the coefficient of variation in DNA measurements can be significantly improved by the addition of RNase and proteinase K. Cell clumping can also be reduced via sonication. Additionally, dyes that only permeate dead yeast cells often require ethanol fixation prior to staining [23,24].

DATA OUTPUT AND INTERPRETATION

Most flow cytometers come with software that will allow simple manipulation of the data collected although more advanced software is also available from several aftermarket vendors. However, for most fermentation analyses this is not required. A means to display the forward scatter (cell size) vs. side scatter (cell granularity) data and a means to overlay different populations will allow analysis of the cell populations. This allows direct comparison of cell size data for different populations – for example comparing a new starter culture with a defined fresh single cell culture to ensure the purity and single cell character of the new culture. Both cell size and granularity can be compared in such analyses to assess health and purity. Additionally, 2-dimensional dot-plots of forward by side scatter data can also be overlaid to allow a similar comparison in two dimensions (**Figure 5**). In each

FIGURE 5 *2-Dimensional Dot-Plot of Forward x Side Scatter Analysis of Cell Size x Cell Granularity.* Typical forward scatter by side scatter dot-plots of yeast populations isolated from two separate active fermentations. Simple assays such as this cell size by cell granularity assessment reveal that 5 distinct cell populations are evident in each fermentation. Each population has been color back-gated to make identification clear.

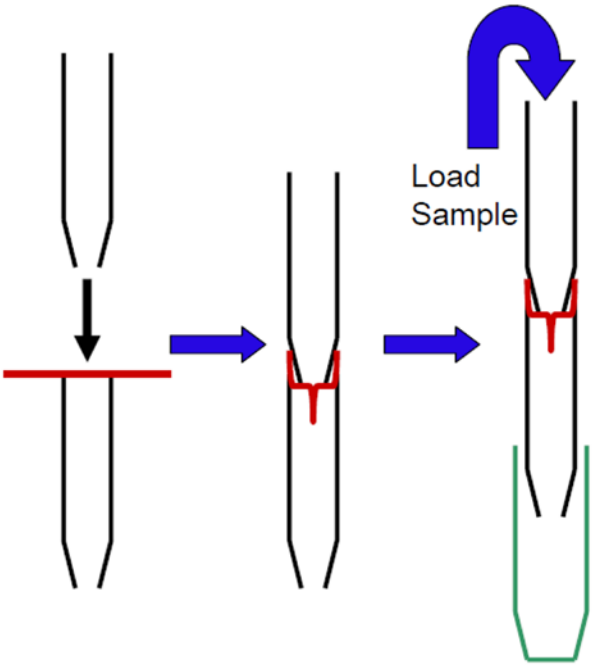


case experience and repeated analysis of similar cultures will provide brewers with the knowledge needed to interpret the results.

SAMPLE PREPARATION

One of the most important aspects in sample preparation when using yeast, especially yeast collected from slurries or propagated from starters, is ensuring that the cell concentration is not too high. When concentrations are too high, the yeast can clog the flow cell. An adequate dilution can generally be accomplished by adding 10 µL of stationary phase yeast culture/slurry to 10 mL of molecular grade water. This results in a 1000-fold dilution. To ensure that the dilution level is adequate, it is recommended that this step is followed by a direct cell count using a hemocytometer or Coulter counter. Following dilution, about 1 mL of this sample is filtered to 60-70 µm using a Nitex filter (Genesee Scientific, San Diego CA). This filtered sample can be analyzed directly for forward and side scatter unless further labeling is required as described below. A portion of the diluted and filtered sample can then be placed in a standard 12 x 75 mm cytometer tube or, if the cytometer will accommodate them, a 1.5 mL microfuge tube for analysis. For high quality analysis it is also essential to filter all liquids, including the sheath fluid and the sample buffer used to suspend the cells, to at least 0.2 µm to ensure that particles are removed from these fluids and are not confused with yeast cells.

FIGURE 6 *Filtration Device.* Simple schematic showing home-made disposable single-use filtration device. Trim the end off one 1000 µL blue micropipette tip and insert into a second tip (black) trapping a trimmed piece of the 20-70 µm filter sheet (red) between them. Load the sample as shown into the top micropipette tip and then force the fluid through the filter with a 1000 µL micropipette into a 1.5 mL microfuge tube (green) ready for flow cytometry.



Once the sample is prepared, additional preparation may be needed depending on the application and if fluorescent dyes are being used. Studies utilizing dyes that only permeate dead yeast cells require that the yeast cells be fixed with ethanol prior to staining. This is easily accomplished by centrifuging aliquots of the sample in 1.5 mL microcentrifuge tubes, removing the supernatant, and resuspending the cells in 1 mL of 70% ethanol to be left overnight at 4°C. For stains that dye DNA, such as PI, SYTOX, and SYBR Green I, the coefficient of variation in DNA measurements can be significantly improved by RNase A/proteinase K treatment and sonication [23,24].

Below we provide a simple procedure for cell staining adapted from the SYBR Green I staining protocol described by Fortuna et al. (2000) [23]. This procedure requires that the cells are fixed beforehand and can be utilized for other stains with some modification.

SIMPLE STAINING PROCEDURE

1. All solutions should be filtered to 0.2 µm before use.
2. At room temperature, centrifuge the 1.5 mL tube containing the ethanol-fixed cells, wash with buffer (50 mM sodium citrate or PBS), then centrifuge again.
3. Remove supernatant and resuspend yeast with 750 µL of buffer.
4. Add 250 µL of 1 mg/mL RNase A solution. It is recommended that 250 µg of enzyme per 10 million yeast cells be used. Incubate for 1 hr at 50°C.
5. Add 50 µL of 20 mg/mL proteinase K solution. It is recommended that 1000 µg of enzyme per 10 million yeast cells be used. Incubate for 1 hr at 50°C.
6. Transfer suspension into 5 mL (12 × 75 mm) polypropylene test tubes. Add the appropriate amount of dye for the sample and incubate. Some dyes like SYBR Green I benefit from an overnight incubation at refrigeration temperatures. Other dyes like PI use a short incubation period of 15 min at room temperature. For dyes sensitive to light, it is recommended that they be protected from light during incubation.
7. To reduce cell clumping, the samples can be sonicated using an ultrasonic probe or bath. Sonication can utilize a few short (~2 sec) bursts or longer intervals from 15 sec to a couple minutes.
8. Finally, each cell suspension sample is passed through a 40-70 µm Nitex filter (Genesee Scientific, San Diego CA) which can be clamped between two blue pipette

tips (the tip of the upper of which has been trimmed short, **Figure 6**) or purchased premade (Flowmi Cell Strainers, Millipore Sigma Inc). Lower filter cut-offs down to 20 µm are also acceptable for yeast. Once filtered, the sample is ready for analysis.

PRACTICAL APPLICATION

Flow cytometry has been widely used to study the cell cycle and related physiological changes that occur in eukaryotic cells using yeast as a model system. In these studies, histograms of fluorescence intensity can indicate phases where the cells are in haploid and diploid states [8,24]. Tied to this, the flow cytometric methods utilizing yeast have contributed to our understanding of the mechanisms involved in budding, cell aging, cell regulation, and the loss of viability [9,10,25]. In industries involving fermentation (i.e., brewing, distilling, wine-making, and biofuel production), these mechanisms are associated with product quality and quantity and fermentation progress. Flow cytometry presents a more rapid assessment over traditional methods taking minutes as opposed to a day or more for single cell culture [26-28]. Food scientists have even used this technology to enumerate and examine both desired and spoilage microbial populations in wine [29,30].

HIGH-SPEED CELL SORTING

Almost from the very beginning, attempts were made to produce flow cytometers that could sort individual cells immediately following analysis. Although they needed to wait for the emergence of more powerful desktop computers, such instruments have been available since the 1980s that were capable of sorting significant numbers of cells and up to 70,000 cells/sec for dedicated biomedical instruments such as the MoFlo (Beckman Coulter). Modern instruments typically sort at approximately 20,000 cells/sec or more with very high purity and low coincidence which occurs when more than one cell is evident in a drop. Such sorting instruments are capable of sorting yeast so, if necessary, such technology can be used to sort pure populations from mixed populations of yeast [7]. These applications are particularly exciting for brewers interested in isolation of wild yeast from the natural environment although care must be taken to ensure that cell clusters are separated efficiently.

FUTURE RESEARCH

NOVEL APPLICATIONS OF FLOW CYTOMETRY TO YEAST

For those brewers interested in isolation of wild yeast from the environment, flow cytometry can offer a rapid and clear answer as to whether new cultures contain yeast. The successful use of selective media to promote the growth of fermentation positive wild yeasts has been reported [5]. However, growth of cultures may contain multiple species or strains of yeast that are alcohol tolerant. Cell sorting can provide a simple and rapid isolation strategy for producing clean discrete cultures that are free of other strains and species. Such approaches are a focus of our research group and should produce cell clones worthy of propagation that are capable of fermentation. While single cell cloning on plates can achieve the same results, the process takes many more days and multiple replatings and runs into the issues associated with sticky cells that are difficult to disperse into single cell populations. Cell sorting employs filtration and then single cell selection during cytometry to avoid this challenge. Thus, it is possible to first identify the presence of complex populations in new isolates and then use cell sorting technologies to isolate and evaluate each population separately.

OTHER SPECIES

Although considerably smaller than most yeast, bacterial cells (at approximately 0.2 µm) are also visible in the flow cytometer. They will appear in the bottom left corner of most dot-plots as small nongranular events. Their presence can be detected and quantified and an assessment of their contribution to the fermenting population determined [31]. While some bacteria are always expected to be present the level of contamination and the specific brewery's tolerance for certain low levels of bacteria can be precisely quantified and assessed. In cases where bacterial species are part of the fermentation complex, the assessment may be more difficult but other means are available to allow and enhance assessment. These include labeling with fluorochromes and altering the way the flow cytometer identifies a single cell counting event from triggering off cell size to fluorescence [32]. Such alterations should be attempted in consultation with the operator and/or director of the flow cytometry laboratory.

SUMMARY

Flow cytometry represents a new rapidly evolving technology that can provide brewers with a novel and remarkably detailed analysis of the yeast populations they are brewing with, including characterization by cell size, granularity, and DNA content, and can offer insights into the viability of individual cells within a culture. The instruments are relatively easy to use and maintain and the cells require only minor manipulations to prepare them for

analysis. Even DNA content can be analyzed with only a few extra fixation and labeling steps. When added to the power of high-speed cell sorting, flow cytometry can provide an extraordinary level of additional analytical knowledge regarding yeast populations compared to that previously available to brewers.

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