Application Note: Epicocconone™ as a Live-Dead Stain for Yeast

Key words: yeast, viability, live/dead stain, flurorescence, epicocconone

Introduction

Assessing the viability of yeast is important in many areas of industrial microbiology. Traditional methods of assessing viability based on counting colony forming unit (CFU) typically take between 24 and 48 hours, and are too slow for practical uses in many industrial situations such as in breweries[1] and in bakeries[2]. Alternatively, direct observation of stained cells by microscopy can be used to rapidly assess yeast viability. The most widely used stain to measure yeast viability is the methylene blue[3]. Although relatively easy to perform the assay produces inconsistent results with both over estimations and underestimations of cell viability under certain circumstances[4]. The methylene blue assay by microscopy is also subjective and tedious, and operators can quickly suffer from fatigue which further impacts on the accuracy of the test.

Viability measurement using flow cytometry and fluorescent vital staining provides a rapid and objective approach to directly measuring yeast cell viability[5, 6, 7]. Various approaches have been adopted to assess yeast cell viability. For example, the DNA intercalating fluorescent red dye, propidium iodide (PI) and the green fluorescent dye oxonol (OX) have been used for measuring membrane integrity and membrane potential, respectively in dead or damaged yeast cells[1, 2, 8]. Fluorogenic esterase substrates, for example, 5-chloromethylfluorescein diacetate (CMFDA) have been commonly used for measuring membrane esterase activity which is only active in intact cells[9].

There is a diversity of factors that lead to the damage or death of cells. To gain a more complete understanding of these events experimenters need a variety of fluorescent dyes that measure different physiological states.

Epicocconone is a pH sensitive fluorescent natural product[10] that changes its emission from red to green in response to pH values < pH 6.5. Here we describe the use of Epicocconone™ to accurately measure changes in cell viability associated with the loss of intracellular pH control in yeast cells. This unique characteristic of Epicocconone™ as a pH sensitive fluorescent dye finds its application to yeast viability studies where homeostasis of the membrane to H^+ is disturbed by an ethanol-induced stressful conditions[11]. Epicocconone™ can be used to simultaneously stain a mixed population of viable and non-viable cells fluorescent orange (for a live population) and green (for a dead population which has lost the ability to maintain intracellular pH). The assay is suitable for analysis either using epi-fluorescence microscopy or flow cytometry.

Materials and Methods

Yeast cells

Baker's yeast *Saccharomyces cerevisiae* strain 167 (MicroBioGen, Australia) was routinely grown in GYP (2% glucose, 0.5 % yeast extract and 1% peptone) medium.
Fresh yeast colonies were inoculated into a 20-mL-cap universal bottle containing 5 mL of 2 % GYP and incubated overnight on an incubated orbital shaker (30 ^{o}C, 180 rpm). Yeast cell suspension (100 µL) was inoculated into 125-mL-cap conical flask containing 25 mL GYP and grown up to an exponential phase (Abs_{640nm} 0.3 – 0.5).

Reagents

1. Epicocconone™, 2.4 mM (1 mg/mL) (FLUOROtechnics, www.fluorotechnics.com/epic.asp) in dimethyl sulfoxide (DMSO)
2. Propidium idodide solution, 1.5 mM (1 mg/mL) (Molecular Probe, Oregon, USA, Catalog No. P-1304) in water
3. bis-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4(3)$) solution, 1.9 mM (1 mg/mL) (Molecular Probe, Oregon, USA, Catalog No. B-438) in ethanol
4. Staining buffer: pH 5 staining buffer made from H_3PO_4 (phosphoric acid; 80 mM), CH_3COOH (acetic acid; 80 mM), and H_3BO_3 (boric acid; 80 mM). The pH of the combined acids was adjusted to pH 5 by adding ~12 mL of 0.2 N NaOH

Equipment

1. Disposable BD Falcon™ polystyrene tube (12 x 17-mm round-bottom test tubes; BD Biosciences Catalog No. 352008)
2. 1.5 mL Microfuge tubes (Sarstedt, Germany)
3. Vortex mixer
4. Centrifuge
5. Microscopic slide and cover slips
6. Epi-fluorescence microscope (Axioskop 2, Carl Zeiss, Jena GmbH, Germany)
7. FACSCalibur (BD Biosciences, Sydney, Australia)

Cytotoxicity test

The cytotoxicity of Epicocconone™ to yeast cells was tested using exponentially grown yeast cells (Abs_{640nm} 0.3 – 0.5). A portion of yeast cells (1 mL-aliquot, 2-5 x 10^6 cells/mL) was harvested in a microfuge and washed in RO water. The cell pellet was re-suspended in 0.5 mL of pH 5 staining buffer. Epicocconone™ solution was directly added to the cell suspension and vortexed gently. Final working concentrations of Epicocconone™ were 1.2, 2.4, 4.8, 9.6 and 24 µM. Cells were allowed to stain at room temperature (dark) for 2 hours. Yeast samples were appropriately diluted in the fresh pH 5 buffer, and the diluted yeast suspension was plated onto 2 % GYP agar medium. Viability of yeast cells was assessed by colony forming units, and compared to yeast samples equally treated in RO water and the pH 5 staining buffer, respectively.

Ethanol treatment and staining method

A portion of yeast cells (1 mL-aliquot, 2-5 x 10^6 cells/mL, Abs_{640nm} 0.3 – 0.5) was treated with varying concentrations (5, 10, 25, 50, and 70 %) of ethanol for 1.5 hours. After washing in sterile RO water, ethanol-treated cells were equally mixed with live cells, and re-suspended in 0.5 mL of pH 5 staining buffer. Epicocconone™ was directly added to the cell suspension (Final working concentration of Epicocconone™ was 4.8 µM). Cells were allowed to stain at room temperature for 30 min (dark). The stained cells were then washed once and re-suspended in the pH 5 staining buffer prior to observation under an epi-fluorescence microscope and analysis by flow cytometry. For comparison with PI (1.5 µM) and OX (1.9 µM), a mixture of ethanol-treated and live yeast cells were prepared as described above, and stained for 10 min at RT.

Epi-fluorescence microscopic observation

Slide samples stained with Epicocconone™ were observed using an epi-fluorescence microscope with a 100-W Hg vapor arc lamp as a light source. The Zeiss filter set 09 with BP 450-490 for excitation filter, FT 510 for beam-splitter, and LP 515 for emission filter was used for all samples. Photomicrographs of the fluorescent cells were taken using Kodak ASA 400 films with ½ seconds of exposure.

Flow cytometry conditions

Flow cytometry was carried out using a FACSCalibur instrument fitted with a 15 mW argon laser (488 nm) and equipped with forward-angle light scatter (FSC < 15°), side-angle light scatter (SSC > 15°) and three fluorescence detectors: Fl1, 515 – 565 nm; Fl2, 565 – 605 nm; Fl3, > 605 nm[12]. Sheath fluid was Osmosol (LabAids). Flow rate was adjusted to keep the total events below 250 s^{-1}. Routinely, 5 × 10^3 cells were analysed per sample. Logarithmic amplification was used throughout, and fluorescence acquisition was gated by light scatter parameters. The FSC detector was set to minimum sensitivity (E-1), and the SSC detector was set to 269 V. Fl1, Fl2 and Fl3 were set to 474V, 320V, and 535V, respectively. Threshold was set on FSC 307 V. All compensation settings were at zero. Data acquired from flow cytometry were analysed using the computer program Windows Multiple Document Interface flow cytometry application (WinMDI; J. Trotter, Salk Institute for Biological Studies, La Jolla, CA, USA). Epi-fluorescence microscopy of sorted cells was carried out as described previously[12]. Cells were assayed for both live and ethanol-treated yeast populations by flow cytometric analysis of Fl-2 (orange fluorescence for live cells) and Fl-1 (green fluorescence for ethanol-treated cells).

Results and Discussion

Cytotoxicity

Epicocconone™ showed no effect on yeast cell viability in experiments that involved exposing yeast cells to concentrations of the fluorophore up to 24 µM for 2 hours (data not shown). Higher concentration or longer exposure times were not tested as this was 5 times the staining concentration and 4 times the staining time.

Microscopy

Epicocconone™ readily permeated and stained yeast cells without any pre-treatment. No background fluorescence was observed even when cells were not washed as epicocconone requires the presence of amines or a hydrophobic environment in order to fluoresce.

Epicocconone™ stained a mixture of ethanol-treated and live yeast cells to yield two distinct populations that appeared orange and green (Figure 1) when viewed using epi-fluorescence microscopy. The yeast population that fluoresced green was shown to be non-viable (> 95 %) and those that fluoresced orange shown to be viable (> 95 %) in experiments where the populations were separated onto membranes using fluorescence activated cell sorting and then cultured on 2 % GYP agar medium.

Flow Cytometry

The effect of ethanol treatment on yeast populations was assessed by flow cytometry using the validated OX and PI methods[5, 8]. These results were compared to Epicocconone™ treatment. Yeast treated with ethanol were mixed with an equivalent quantity of untreated cells and analysed by flow cytometry.

Treatment with 5 or 10 % of ethanol had no effect on yeast vitality (Figure 2) measured by either PI, OX, or Epicocconone™, but at 25 % of ethanol membrane potential and membrane integrity are affected as evidenced by the appearance of a second population of highly fluorescent cells either stained by PI (A3-R1) or by OX (B3-R1). Similarly, Epicocconone™-staining revealed a second population (C3-R1) that was green fluorescent, indicating a loss of cytoplasmic pH control. Live cells with functioned proton pumps remained orange fluorescent. The 34 % of the 25 % ethanol-treated yeast cells had impaired proton pump activity. This population (C3-R1) was gated, sorted and plated and shown to have also lost their ability to form colonies.

At higher concentrations (50 and 70 %) of ethanol, virtually all the yeast cells were non-viable (> 95 %) and the results were consistent across all three stains (PI, A4-5; OX, B4-5; Epicocconone™, C4-5).

Epicocconone™ freely diffuses across membranes without aid of permeabilization steps. The live / dead Epicocconone™ assay depends on measuring the intracellular pH in response to ethanol treatment[11]. Live yeast cells maintain a constant internal pH of 7.0 and thus stain in fluorescence orange at pH values > 6.5. Ethanol-treated yeast cells lose cytoplasmic membrane integrity or potential and thus their intracellular pH reflects the pH of the external environment (pH 5 in this case). Thus at low pH, Epicocconone™ stained ethanol-treated yeast cells fluoresce green.

Conclusion

Due to the changes in fluorescent emission at different pH values, staining with Epicocconone™ provides a simple method for determining intracellular pH in yeast cells. Here this measurement of intracellular pH has been used in physiology studies to assess the viability of ethanol treated yeast cells.

Epicocconone has the following useful properties:

- **Non-toxic**: Epicocconone-staining does not affect viability of yeast during staining procedures even at 5 times the working concentration.
- **One fluorophore for two-color-differential staining:** Epicocconone does not need another fluorophore to differentially stain dead or live yeast populations as epicocconone is pH sensitive.
- **Intracellular pH indicator:** Epicocconone can be used to measure the intracellular pH of cells by comparing green vs. orange fluorescence, which is linear between pH 5 and 8. Thus it may be possible to measure intracellular pH of organelles (e.g. lysosomes) or cells using Epicocconone™.

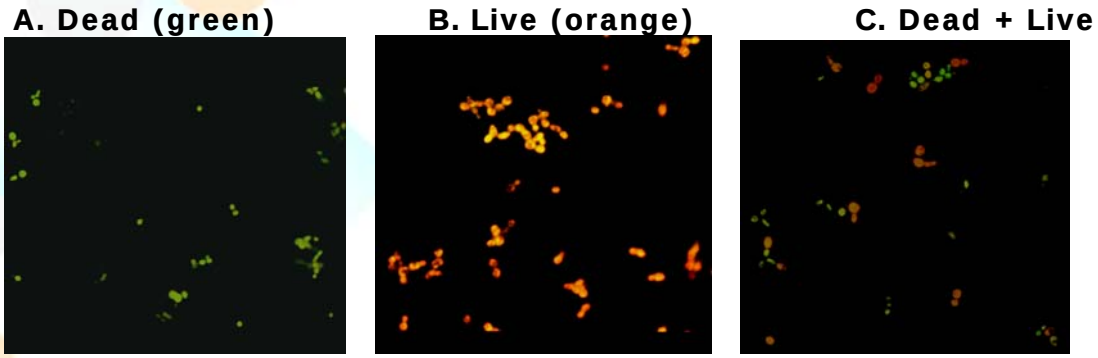

Figure 1. An example of dual colour- Epicocconone™ staining of yeast cells treated with 70 % of ethanol. A, ethanol-treated yeast cells stained with Epicocconone™ in pH 5 staining buffer; B, live yeast cells stained with Epicocconone™ in pH 5 staining buffer C, A mixture of ethanol-treated and live yeast cells (1:1) stained with Epicocconone™ in pH 5 staining buffer. The Zeiss filter set 09 with BP 450-490 for excitation filter, FT 510 for beam-splitter, and LP 515 for emission filter was used.

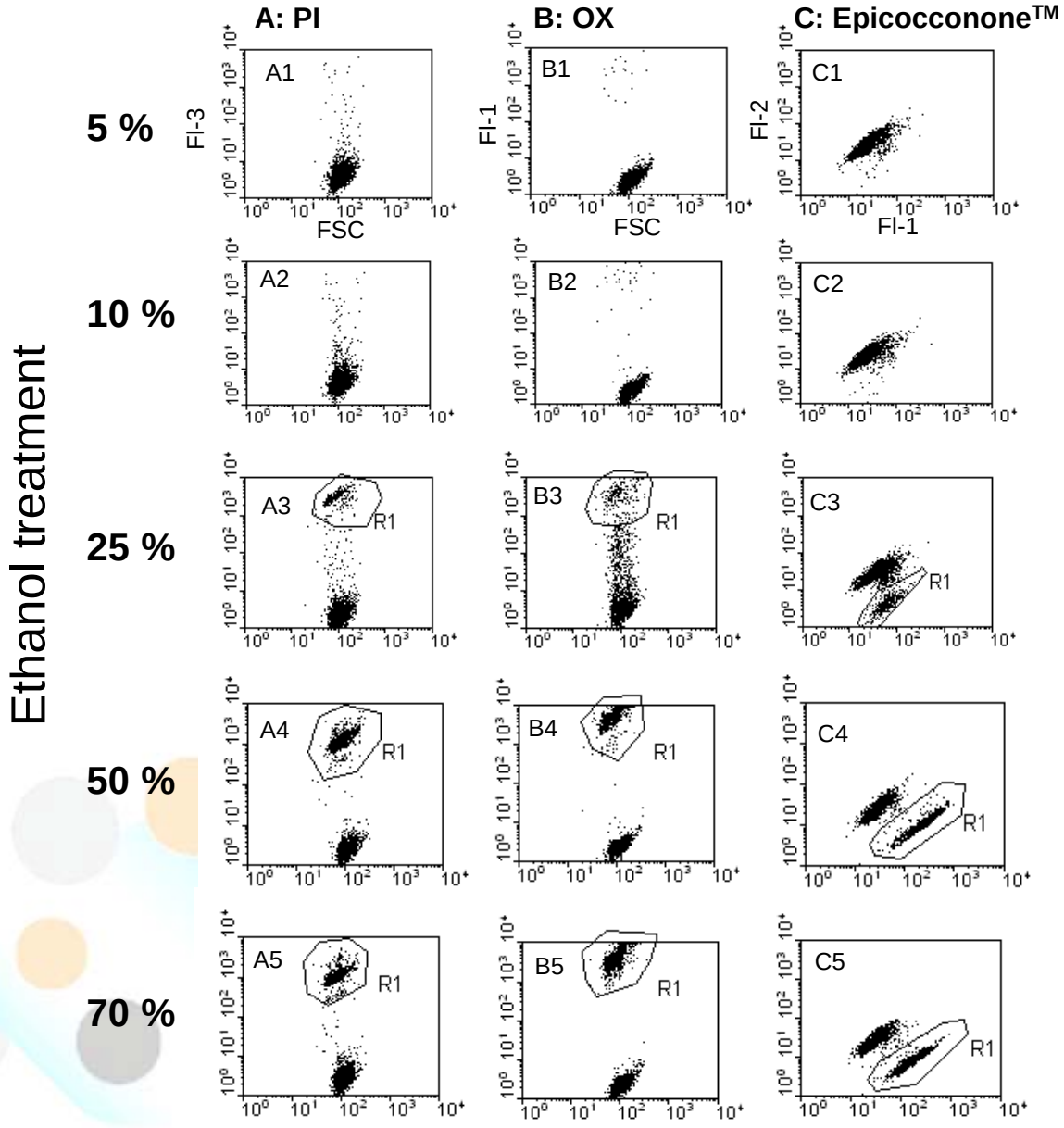

Figure 2. Flow cytometry analysis of yeast cells treated with ethanol and stained with either PI, OX or Epicocconone™. Column A, PI-staining; B, OX-staining; C, Epicocconone™-staining. Row 1, 5 % ethanol treatment; Row 2, 10 % ethanol; Row 3 (A3-C3), 25 % ethanol; Row 4, 50 % ethanol; Row 5, 70 % ethanol. All gains were logarithmic. FSC, forward-angle light scatter; SSC, side-angle light scatter; FL1, green fluorescence channel; FL2, orange fluorescence channel; FL3, red fluorescence channel (expressed as volts). R1 indicates the gated region of yeast populations, confirmed to be non-viable by CFU counting.

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