RESOLUTION OIV-OENO 713A-2025

YEAST CELL COUNTING USING FLOW CYTOMETRY IN GRAPE MUSTS AND WINES

THE GENERAL ASSEMBLY

CONSIDERING that to properly manage alcoholic fermentation and secondary fermentation in sparkling wines, rapid and accurate methods capable of providing information on the numbers and physiological state of the oenological yeast population should be available,

CONSIDERING that, currently, the majority of OIV methods regarding microbiological analysis are based on Petri dish counting, a technique that is robust and effective but that requires long incubation times which are sometimes incompatible with the speed of fermentation processes,

CONSIDERING that, nowadays, flow cytometry is an analytical technique that is widely used in various sectors of the biotechnology and agri-food industry; there is ample evidence of its robustness and reliability and numerous technological solutions are available on the market suited to different production contexts,

DECIDES, to adopt the following microbiological analysis method for grape musts and wines and to include in Compendium of International Methods of Wine and Must Analysis:

YEAST CELL COUNTING USING FLOW CYTOMETRY IN GRAPE MUSTS AND WINES

Method [OIV-MA-[corresponding reference]

Type IV method

1. YEAST CELL COUNTING USING FLOW CYTOMETRY IN GRAPE MUSTS AND WINES

2. Scope

Method for quantifying viable, stressed (permeable membranes) and dead yeast cells.

This method using double labelling does not allow the quantification of viable

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metabolically inactive cells (impermeable membranes, VMI).

The method can be applied to wines, musts, musts during alcoholic fermentation, and foam captures.

Quantification limits depend on the performance of the equipment used and the method of sample preparation.

3. Definitions

FLOW CYTOMETRY. Flow cytometry is a technology that provides rapid and multi-parametric analysis of single cells in solution. Flow cytometer utilizes lasers as light sources to produce both scattered and fluorescent light signals that are read by detectors such as photodiodes or photomultiplier tubes. These signals are converted into electronic signals that are analysed by a computer. Cell population can be differentiated and/or characterized on the basis on their fluorescent or light scattering characteristics.

FORWARD SCATTER (FSC). Signal generated by the scattering of light by the particle (cell). By convention it is recorded at 180° from the light source and can be directly related to particle size.

SIDE SCATTER (SSC). Signal generated by the scattering of light by the particle (cell). By convention it is recorded at 90° from the light source and can be directly related to the structural complexity of the particles.

FLUORESCENCE CHANNEL (FLx). Signals generated by the emission of fluorescence due to the fluorochromes associated with the particles by means of suitable colours. The different emission wavelengths are separated by optical filters and, by convention, numbered progressively (FL1, FL2, etc.).

COMPENSATION. The process of correcting of fluorescence spill over, by removing the signal of any given fluorochrome from all detectors except the one devoted to measuring that dye. Typically, is applied at the channels FL2 or FL3, to eliminate the contribution of fluorochrome that has the maximum of emission peak in FL1 channel.

VOLUMETRIC COUNT. Measure of number of events (cells) in a fixed volume of sample, usually measured by two electrodes placed at different levels in the sample cuvette. Some alter solutions, i.e., the use of a volumetric pump is acceptable.

EVENT. An event is defined as the detection of a single particle that passes through the instrument's laser beam(s). Each event corresponds to a set of measurements of different optical and physical properties of this particle. It is up to the operators to demonstrate that an event can be associated with a single cell, by checking the absence of clumped cells (doublets, triplets, etc.).

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4. Principle

A cell suspension, obtained by appropriate decimal dilution of the sample, is analysed using flow cytometry in volumetric mode, after marking with fluorescent dyes capable of differentiating between cells having enzymatic activity (live) and cells with compromised cytoplasmic membrane (dead). Also, in some cases is possible to identify a 3rd sub population of cells having metabolic activity, but altered permeability of cell membrane, this sub population is generally considered as stressed, but viable, cells.

The method proposed here is a generic method using a single blue laser and 2 fluorochromes. More sophisticated methods, using multi-laser cytometers, and multiple markings are possible.

2 types of fluorochromes are used in this method:

- Propidium iodide (PI): this is an intercalating agent for nucleic acids (DNA or RNA). It only penetrates cells with permeable plasma membranes. It is accepted that these are primarily dead cells, or those exhibiting membrane stress (for example under the effect of ethanol). Its excitation peak is between 520 and 550 nm, and the maximum fluorescence emitted is between 610 nm to 630 nm. Cells marked IP(-) are therefore considered viable, and IP(+) cells as dead or presenting permeable plasma membranes.
- 5(6)-Carboxyfluorescein diacetate (cFDA). This is a cell-permeant esterase substrate acting as a probe of metabolic (esterase) activity. During hydrolysis by intracellular esterases, this acetoxymethyl ester produces carboxyfluorescein, whose excitation peak is at 498 nm, and the fluorescence emission maximum is at 516 nm. Its emission spectrum extends to 650 nm, which may require compensation on other channels (FL2). Cells marked cFDA(+) are therefore considered metabolically (esterase) active, and cFDA(-) cells as metabolically inactive.

Table 1: summary of interpretations based on fluorochrome responses

Quadran	**IP(-)**	**IP(+)**
cFDA(-)	Not interpretable in this method	Dead

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cFDA(+)	Viables and active	Active cells with altered plasma membrane permeable to IP (stressed cells)

Note 1: A population of IP(-) cells is defined which reflects membrane integrity and cFDA(-), which reflects non-detectable metabolic activity.

Note 2: In this method, the cells are not dissociated from possible background noise. The IP(-) quadrant, cFDA(-), is therefore not interpretable.

5. Reagents and materials

Laboratory glassware, flow cytometry cuvettes and sheath fluid, where required by the instrument.

Test tubes (16x160 mm or similar) containing 9 mL of Phosphate-buffered saline (PBS) filtered through a 0.2 µm porosity filter (pH 7.4).

5(6)-Carboxyfluorescein diacetate (Powder) (cFDA ; CAS 124387-19-5)

Propidium Iodide, (powder) 95 % (IP ; CAS 25535-16-4),

Dimethyl sulfoxide pure liquid form (DMSO ; CAS 67-68-5)

Pure culture of *S. cerevisiae* (e.g. ATCC 9763 strain) with a nominal concentration of 10^5 cell/mL.

NaCl solution 8,5 g/L in water, sterilised by filtration or autoclave.

cFDA solution in DMSO or acetone, 0.1 mg/mL.

IP solution in DMSO or aqueous solution, 1 mg/mL.

Note 1: The dilution of fluorochromes is done in DMSO. It is possible to do this in acetone. However, it appears that the acetone/DMSO mixture produces more background noise on the cytometer, and it is recommended to use only DMSO.

6. Apparatus

Flow cytometer equipped with a 488 nm laser (50 mW) and FSC, SSC, FL1 (530 nm), FL2 (630 nm) and FL3 (670 nm) optical parameters.

Vortex type mixer.

Laboratory glassware.

0.2 µm sterilizing laboratory filter (preferably made of cell acetate which limits background noise).

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Flow cytometry plate.

1 mL and 0.2 mL micropipettes with sterile tips.

Analytical balance with ± 0.01 g accuracy.

Laboratory centrifuge.

Note: Sterility is not essential, however, it is recommended to maintain a high degree of hygiene and use sterile equipment and reagents.

7. Sampling (preparation of the sample)

Reference is made to method OIV-MA-AS4-01 Microbiological Analysis of Wines and Musts - Detection, Differentiation and Counting of Micro-organisms in the COMPENDIUM OF INTERNATIONAL METHODS OF ANALYSIS (Resolution OIV/OENO 206/2010) regarding wines and musts.

It is recommended to homogenize the whole mass of wine before the collection of the sample, and the sample prior the analysis, to ensure a homogenous dispersion of cells in the wine.

8. Procedure

Flow cytometry analysis is immediate, in general in modern apparatus it last less than 3 minutes; there is little risk of contamination due to sample handling outside the laminar flow hood or during cytometric reading. However, it is advisable to maintain a high degree of hygiene and to use sterile equipment and reagents.

The procedure is given as an example. Variations may be introduced by the laboratory. The flow cytometry technique requires to constantly adapt the dilutions according to the equipment used, and the microbiological loads of the products analysed.

SETTING UP THE FLOW CYTOMETER

Switch on the flow cytometer and wash the device to prevent interference with the analysis. Set acquisition channels FSC, SSC, FL1 (acquisition at 530 nm), FL2 (acquisition at 630 nm) and FL3 (acquisition at 670 nm) on a logarithmic scale.

The flow cytometer can be considered ready for analysis when there is no evidence of events capable to interfere with the acquisition of sample in the FSC channel when reading a sample containing only sheath fluid.

Read a pure culture of *S. cerevisiae*, taking care to adjust the voltages of the FSC and SSC channels, if necessary, so that the signal peak in the two FSC/SSC channels is well separated from background noise. If flow cytometry allows it, it is possible to eliminate the contribution of background noise by acting on the threshold of the FSC

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parameter. Construct a dot plot by integrating the FSC and SSC signals, and locate the region containing the yeast cell population, identifying it with a specific gate (Figure 1).

SAMPLE STAINING

Wine or must, preparation for a yeast population > 10^2 cell/mL

Dilute the sample between 1/10th and 1/100th (or even more), depending on the performance of the equipment used, and the microbiological load, in the NaCl solution.

Place 980 µL of diluted sample in a cytometry cuvette, according to the instrument specifications.

Carry out the marking with, for example, 10 µL of cFDA solution and 10 µL of IP solution, in 980 µL of diluted wine or must.

Incubate for approximately 10 minutes at room temperature, in the dark.

At the end of the incubation homogenise and read the sample, after setting the flow cytometer to volumetric reading mode.

Wine or must, preparation for a yeast population < 10^2 cell/mL (e.g. packaged wine).

Centrifuge 50 mL of sample at 4500 rpm, 8 min, approximately.

Eliminate the supernatant, collect the centrifugation pellet.

Take the pellet in, for example, 10 mL of NaCl solution.

Carry out the marking with, for example, 10 µL of cFDA solution and 10 µL of IP solution, in 980 µL of rediluted pellet .

Incubate for approximately 10 minutes at room temperature,

in the dark.

At the end of the incubation homogenise and read the sample, after setting the flow cytometer to volumetric reading mode.

Note 1: The final concentration of cFDA in the labelled sample is around 2 to 5 mg/L. The final concentration of IP in the labelled sample is of the order of 3 to 10 mg/L.

Note 2: There is no importance on the order of the fluorochromes contributions. They can be brought at the same time in a solution containing the two fluorochromes.

Note 3: The marking is generally stable for 1 hour or more.

FLOW CYTOMETRIC ANALYSIS

Read the sample, taking care to adjust the instrument settings to ensure the yeast population falls within the gate previously identified with the pure *S. cerevisiae* culture.

Adjust the voltages of the FL1 and FL2 channels to separate better the emission peak

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of sample from the background noise (autofluorescence). To avoid aberrations due to the FDA emission spectrum, which could also intercept the FL2 channel, apply the appropriate level of compensation to subtract the contribution of FL1 from FL2. If appropriate, the channel FL3 would be used instead that FL2 channel to allows a better discrimination of fluorescence signals related to live (FL1) and dead (FL2 or FL3) cells. Perform a volumetric count of the cells present in the sample. Integrate the FL1 and FL2 (or FL3) channels in a dot plot to better visualise the separation of the cell populations (Figure 2). Consider as live cells the events collected in the FL1 channel and coming from the gate containing the yeast population, identified in the dot plot of the FSC and SSC physical parameters. Consider as dead cells the events collected in the FL2 (or FL3) channel and coming from the gate containing the yeast population, identified in the dot plot of the FSC and SSC physical parameters. Consider as stressed, but viable, cells the population of events that remain positive on both channels (FL1 and FL2 or FL3) after appropriate compensation of signals.

9. Calculation

After completing the volumetric count, note the number of live, dead, and damaged cells counted per unit of volume or weight, as a function of the volume sampled by the flow cytometer, considering the decimal dilutions performed. As this is a direct cell count, it is possible to express the data in “cells/mL” or by “g” while checking for the absence of doublets or triplets.

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Figure 1. FSC/SSC dot plot showing the gate of the population of events corresponding to S. cerevisiae (yeast) cells present in a sample with a concentration of 10^5 cell/mL.

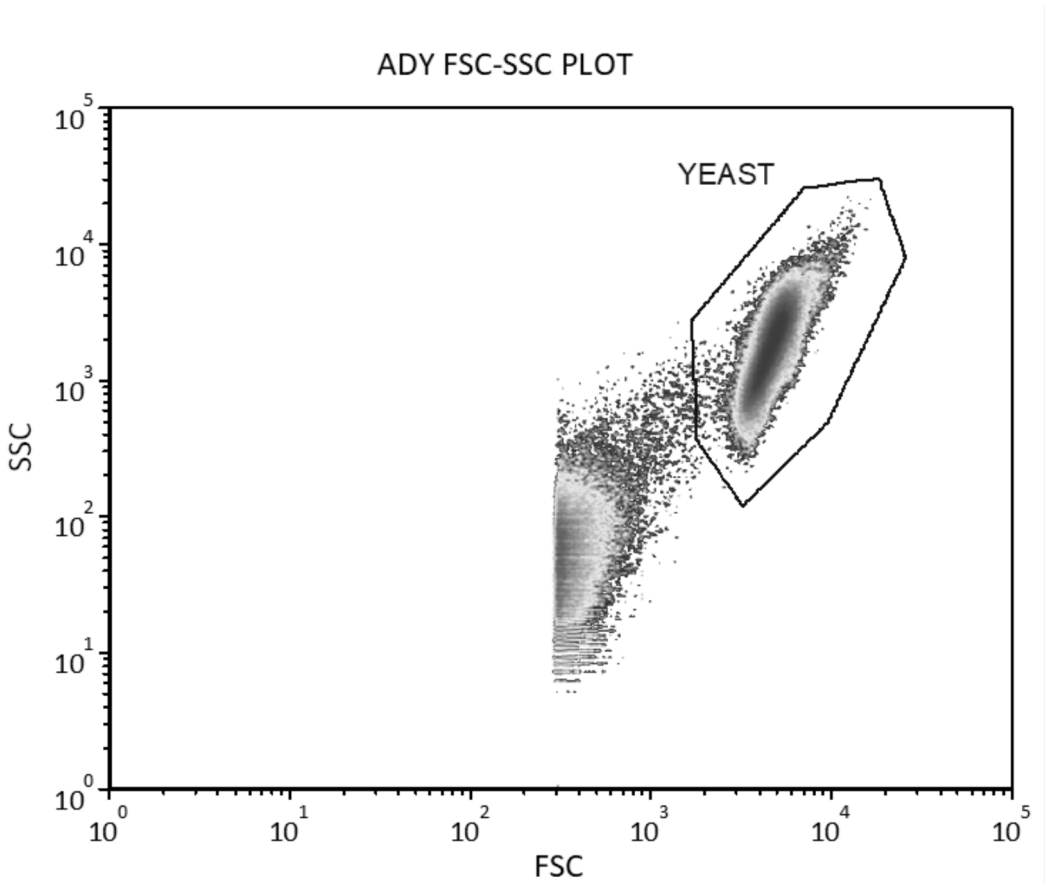

Figure 2. FL1 (530 nm)/FL2 (630 nm) dot plot showing the gates containing the population of events corresponding to live, dead and stressed, but viable, S. cerevisiae cells.

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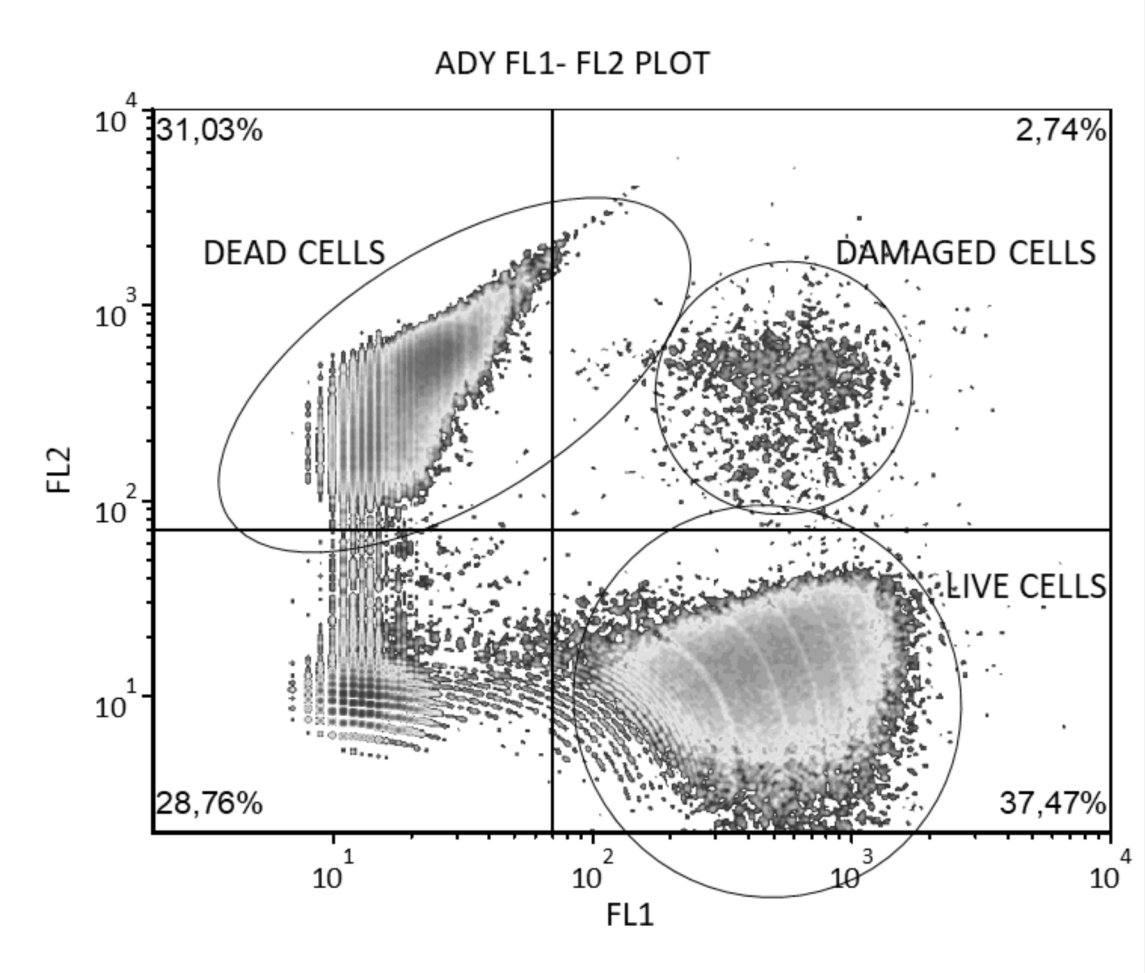

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