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Portable, Cost-effective, and Rapid Yeast Cell Concentration and Viability Measurement using Lensless On-chip Microscopy and Support Vector Machine Classification

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A thesis submitted in partial satisfaction

of the requirements for the degree

Master of Science in Bioengineering

by

Alborz Feizi

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ABSTRACT OF THE THESIS

Portable, Cost-effective, and Rapid Yeast Cell Concentration and Viability Measurement using Lensless On-chip Microscopy and Support Vector Machine Classification

by

Alborz Feizi

Master of Science in Bioengineering

University of California, Los Angeles, 2016

Professor Aydogan Ozcan, Chair

The monitoring of yeast cell concentration and viability is essential for beer-brewing and biofuel production industries. However, the current methods of measuring viability and concentration are relatively bulky, costly, and/or tedius. We have developed an Automatic Yeast Analysis Platform (AYAP) that performs portable, cost-effective, and rapid measurement of these conditions using a lensless microscope based on partially-coherent in-line holography. This microscope weighs 70 g, has dimensions of 4 × 4 × 12 cm, and communicates with a touch-screen user interface. The user interface utilizes a Support Vector Machine (SVM) classification algorithm to automatically measure concentration and viability of yeast samples stained with methylene blue. AYAP's measurements agreed well with gold-standard fluorescence-based manual counting measurements, demonstrating AYAP's dynamic concentration range of 1.4×10^5 to 1.4×10^6 cells/mL. This range of cell densities is ideal for various fermentation-based industries.

The thesis of Alborz Feizi is approved.

Warren S. Grundfest

Dino Di Carlo

Aydogan Ozcan, Committee Chair

University of California, Los Angeles

2016

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1 Introduction[1]

Baker's yeast, *Saccharomyces Cerevisiae*, has a number of applications in industries such as baking, beer-brewing, and ethanol fuel production [2–6]. The measurement of yeast concentration and viability is crucial in optimizing the fermentation process for these industrial applications [7–9]. A rapid, cost-effect, and mobile method to measure viability and concentration would greatly assist these industries in increasing their efficiency and productivity.

Nonetheless, the existing methods of measuring concentration and viability are costly and/or laborious. A tedious and time-consuming method [10] of measuring these conditions entails the use of a counting hemocytometer and a bench-top microscope in order to manually count the number of viable and non-viable cells in a yeast sample [7]. As a substitute, there exists imaging platforms that automatically tally the number of live and dead cells by utilizing motorized assemblies and combining fluorescence and bright-field microscopy [11]. However, these components reduce the mobility and increase the cost of such platforms. Another bulky method of measuring viability and concentration is via flow-cytometery [12], which requires relatively expensive equipment operated by a trained technician. Thus, our goal was to develop an alternate method of viability and concentration measurement, one that is both cost-effective and portable.

This paper details an Automatic Yeast Analysis Platform (AYAP), which measures concentration and viability utilizing a portable and cost-effective lensless microscope [13–16] and a touch-screen interface that uses a Support Vector Machine Classification [17,18] algorithm to determine the concentration and viability in yeast samples mixed with a viability stain. As seen in figure 1a, the lensless microscope contains a partially coherent illumination source consisting of a light-emitting diode (LED), a 0.1 mm multimode optical fiber, and a band-pass

[1] This manuscript has been submitted to a peer-reviewed journal for publication [1].

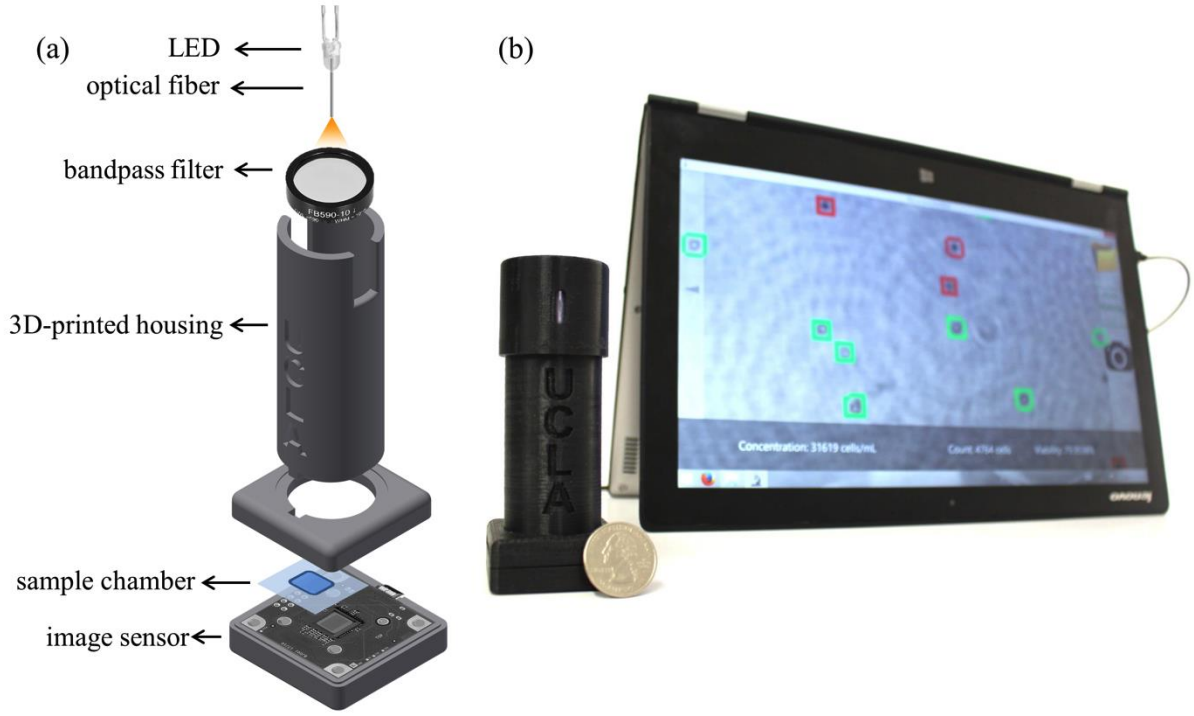


Figure 1 | (a) Lensless microscope – An LED with a peak wavelength of 590 nm is coupled to an optical fiber, which is placed in front of a bandpass optical filter with a 4 nm bandwidth. The sample is directly placed on a CMOS sensor, positioned 6 cm away from the illumination source. (b) Touch-screen interface receives the hologram captured by the lensless microscope. This interface uses a SVM classification algorithm to back-propagate the hologram to the object plane and classify live and dead cells indicated with green and red labels respectively. This figure has been submitted to a peer-reviewed journal for publication [1].

optical filter. In this microscopy setup, the yeast sample is positioned on top of a Complementary Metal-Oxide Semiconductor (CMOS) image sensor chip, which captures the interference between the light diffracted by the yeast cells and the partially coherent background illumination. This holographic interference pattern is used to form a large field-of-view (FOV) image of the object plane. The user interface utilizes the Support Vector Machine Classification algorithm to perform auto-focus and classify stained and unstained cells. Our on-chip microscopy platform has a 22.5 mm^2 FOV, which is an order of magnitude larger than a conventional 10x microscope objective lens FOV, allowing for rapid and accurate viability and concentration measurement.

We compared the automatic concentration and viability percentage measurement via AYAP with manual counting and measurement of concentration and viability percentage using a gold-

standard fluorescent exclusion stain. Regression analysis showed no significant difference between the two methods, demonstrating our mobile platform's ability to accurately measure concentration and viability within the wide concentration range of 1.4×10^5 to 1.4×10^6 cells per milliliter.

2 Materials and Methods

Yeast Viability Staining

A Methylene blue solution (0.1% w/v) was mixed with baker's yeast, *Saccharomyces Cerevisae*, in a 1:1 volume ratio. Methylene blue penetrates yeast cells; however, viable cells contain enzymes that reduce methylene blue to a colorless compound [19]. Therefore, nonviable cells are stained blue and viable cells remain colorless, allowing for measurement of yeast viability.

Microfluidic Counting Chamber

Acetal Polyoxymethylene material with acrylic adhesive (CS Hyde, 45-3A-1) was cut in the shape of square using a laser-cutter (VersaLaser VLS 2.30) and used as a spacer. This material was attached to a coverslip (thickness: 0.13-0.17 mm) using the acrylic adhesive. Another coverslip of the same thickness was placed on top of the spacer material, leaving a small opening for pipetting yeast cells into this microfluidic chamber. When dispersion of yeast cells through the counting chamber is complete, the top cover slip is slightly shifted to close the small opening and reduce evaporation.

Portable and Cost-effective Lensless Microscope

Device housing was 3D printed (3D printer: Stratasys, Dimensions Elite) using acrylonitrile butadiene styrene (ABS) material. An LED (λ = 590 nm, Kingbright, WP7113SYC/J3) is

coupled to a multimode optical fiber (100 μm core diameter, Thorlabs, AFS-105/125Y) to improve spatial coherence. A band-pass optical filter (4 nm bandwidth, Thorlabs, FB590-10) is used to improve temporal coherence. The microfluidic counting chamber is directly positioned on top of the CMOS sensor (ON Semiconductor, MT9J003STM), which is approximately 6 cm away from the illumination source (see figure 1).

The holograms captured by the image sensor are transformed to the spatial frequency domain via a fast Fourier Transform (FFT). Next, a phase factor, which is a function of the medium refractive index, the wavelength, and the propagation distance, is multiplied with the angular spectrum. Lastly, it is transformed back to the spatial domain using a inverse Fourier Transform, resulting in the back-propagated image of the yeast cells [16,20,21].

Support Vector Machine Classification of Live and Dead Cells

A Machine Learning Support Vector Machine (SVM) Classification algorithm [17,18] is used for auto-focus and identification of live and dead cells. The trained data was populated with 260 stained and 260 unstained cells obtained from two individual yeast samples. The viability of each cell was confirmed via images captured with a high-resolution bench-top microscope (10x objective lens, 0.3 NA). For each cell candidate, 10 spatial features, such as mean intensity, area, and perimeter were extracted from the back-propagated image. The hologram captured by the CMOS sensor is digitally divided into a 2 x 3 grid, resulting in 6 sub-FOVs. Independent processing of each sub-FOV allows us to minimize the effects of sample tilting and variance in the thickness of the microfluidic counting chamber. Each sub-FOV is subject to an auto-focus algorithm based on the trained SVM model (see figure 2). This algorithm back-propagates the captured hologram to multiple distances (z_2) from the image sensor. The cell candidates at each back-propagation distance are fed into the SVM classification model. The signed distance

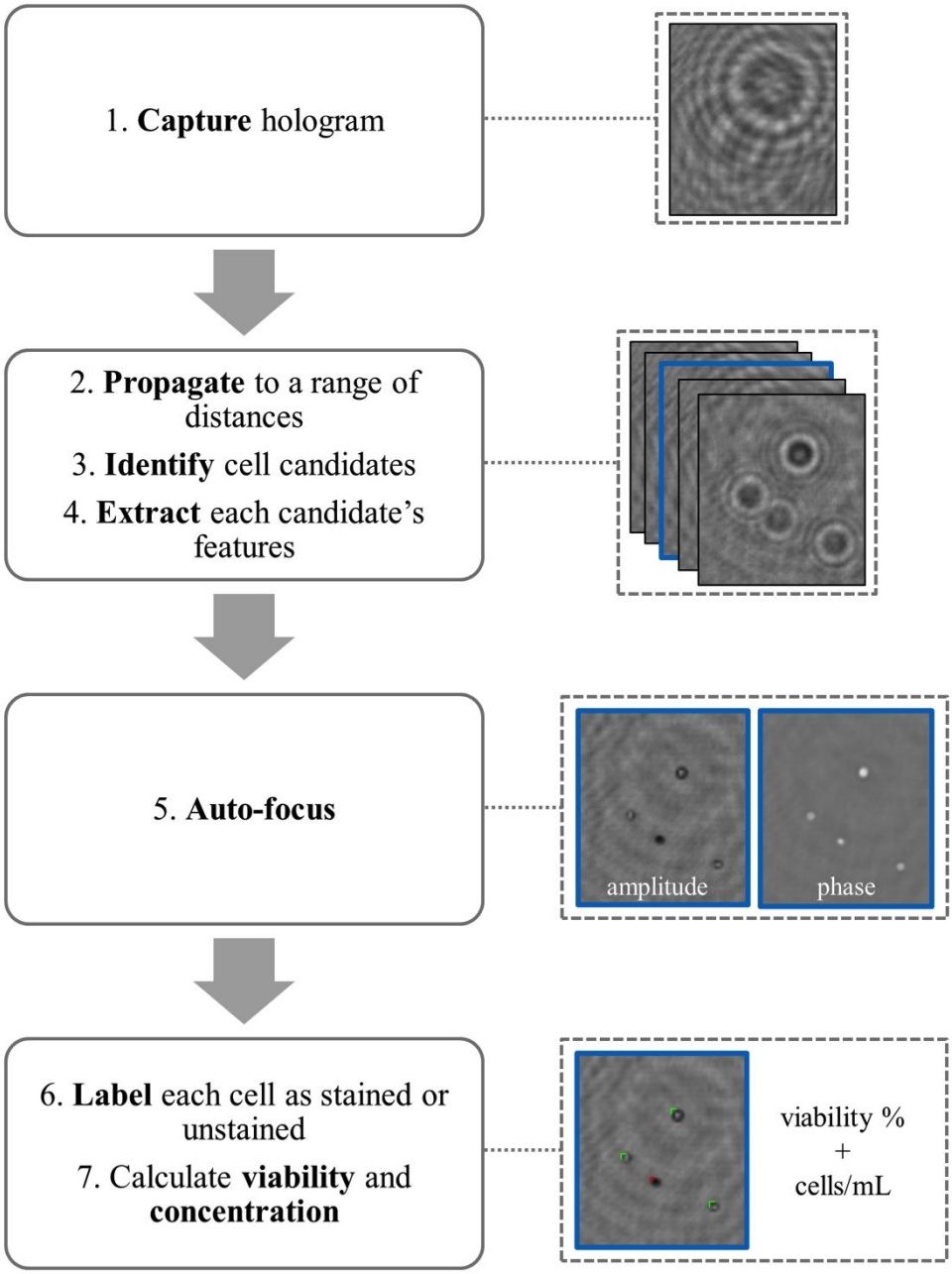


Figure 2 | The holographic interference pattern of yeast cells is captured by the CMOS image sensor and propagated to a range of distances from the image sensor. The cell candidates in each propagated image are identified and fed into a pre-trained SVM classification model. Ten features including area, perimeter, and standard deviation are extracted from each candidate. Next, an SVM score, the signed distance from the decision boundary, is assigned to each candidate. The propagation distance z_2 with the greatest mean SVM score is chosen as the optimum distance. Finally, all cell candidates are labeled using the same SVM classification algorithm and the viability and percentage are calculated. This figure has been submitted to a peer-reviewed journal for publication [1].

s_i $(i = 1, \dots, N)$ from the decision boundary (classification score) is calculated for each cell candidate, where N is the total number of cell candidates. The propagation distance with the

greatest absolute mean SVM classification score is displayed to the user and used for detection of live and dead cells using the same SVM classifier. This auto-focus criterion can be described in a mathematical formula:

$$\underset{z_2}{\text{argmax}} \; f(z_2) = \frac{\sum_{i=1}^{N} |s_i|}{N}$$

After performing auto-focus, majority of the twin-image related artifacts, dust particles, and cell clumps are removed based on a SVM score threshold. These objects are classified as live or dead but they have relatively small distances from the decision boundary: $|s_i|$. The same SVM score threshold is applied to all measurements. The remaining cell candidates are labeled as green or red based on their live or dead classification.

Touch-screen User Interface

Touch-screen interface was developed to back-propagate captured holograms and display concentrations and viability percentages. This interface runs on a tablet-PC (Lenovo Yoga 2) that is connected to the portable lensless microscope via USB ports. The user has the ability to directly capture a hologram from the image sensor or load a previously saved hologram (figure 3a). Next, the hologram is fed into the SVM classification and the optional auto-focus algorithms described earlier. The entire analysis using auto-focus can take 5-10 minutes depending on the number of cell candidates within the FOV. However, when using the same batch of coverslips, z_2 distances are consistent from sample to sample. Therefore, we can run the digital auto-focus algorithm once and use the same list of z_2 (per tile) for all the samples. The entire analysis without digital auto-focusing takes less than 30 seconds to complete. The cell candidates classified as live and dead are displayed on the image using green and red labels (figure 3c). The user may digitally zoom the image in order to inspect the classified cells (figure 3d). The interface also contains three separate bar graph that display total cell concentration, unstained

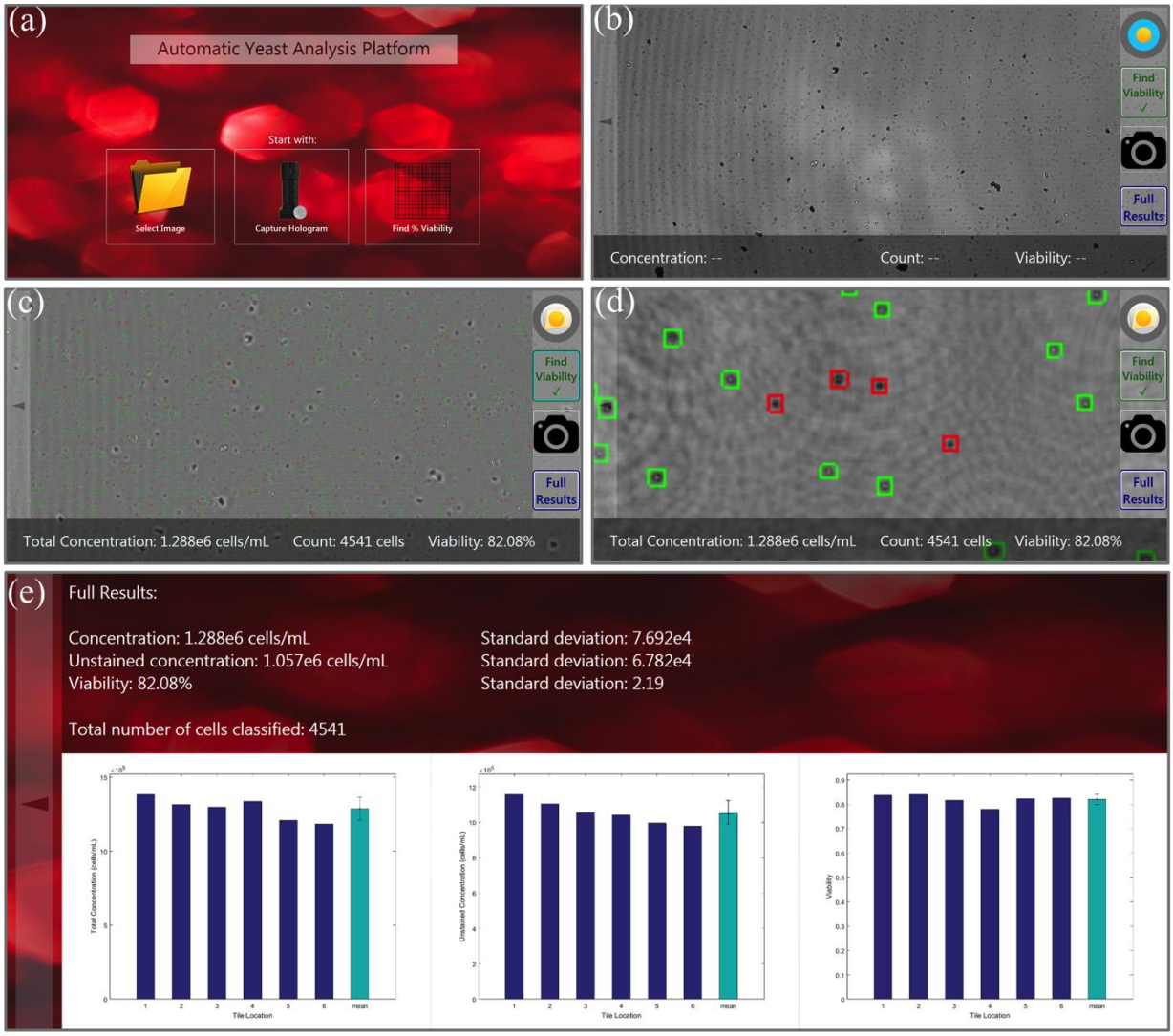


Figure 3 | (a) The user may capture a hologram directly from the lensless microscope or load a previously saved hologram. (b) The large FOV is divided into six tiles. This FOV can be analyzed using auto-focus or using a list of per-tile propagation distances obtained from a previous experiment. (c) SVM classification model is used to label live cells green and label dead cells red. (d) The user may zoom the image to inspect labeled cells. The concentration and viability percentage information is also displayed at the bottom of the page. (d) Per-tile statistics is displayed on this page. These statistics include total concentration, unstained concentration, and viability percentage along with mean values and standard deviations. This figure has been submitted to a peer-reviewed journal for publication [1].

cell concentration, and viability percentage for each tile within the 2x3 grid (figure 3e).

Furthermore, it displays the mean concentration and viability in addition to standard deviation within the tiles.

3 Results and Discussion

An established method of measuring cell viability is exclusion staining. In this method, dead cells are stained and the ratio of unstained cells to the total number of cells represents the viability ratio [22–24]. Methylene blue [19,25] is one of the available exclusion stains [26–28] used in industry. This stain may be stored at room temperature and has a relatively low toxicity to humans [29]. Conventional methylene blue exclusion staining has two important disadvantages. Firstly, long exposure of cells to this stain may result in underestimation of viability ratio due to false positives [28]. Secondly, this staining method is more prone to operator subjectivity when compared to fluorescence-based staining [30,31]. However, these reported disadvantages do not affect our computational microscopy platform. Our platform captures a wide FOV hologram (see figure 4) of the sample in less than 10 seconds, reducing false-positives. Furthermore, our SVM classification algorithm removes subjectivity from the live/dead identification process. Therefore, methylene blue is a suitable stain for our portable and rapid platform.

We compared automatic concentration and viability measurement using methylene blue with manual concentration and viability measurement of yeast samples stained with a fluorescence exclusion stain – propidium iodide. We prepared yeast samples at various concentrations and viabilities for the purpose of this comparison. Each sample was divided into two sub-samples of equal volume, one sub-sample stained with methylene blue and the other sub-sample stained with propidium iodide. A single hologram of the methylene blue sample was captured using AYAP and divided into six sub-FOVs processed by the back-propagation and SVM algorithms individually. On the other hand, 4-5 microscope (10x objective lens, 0.3 NA) images of the

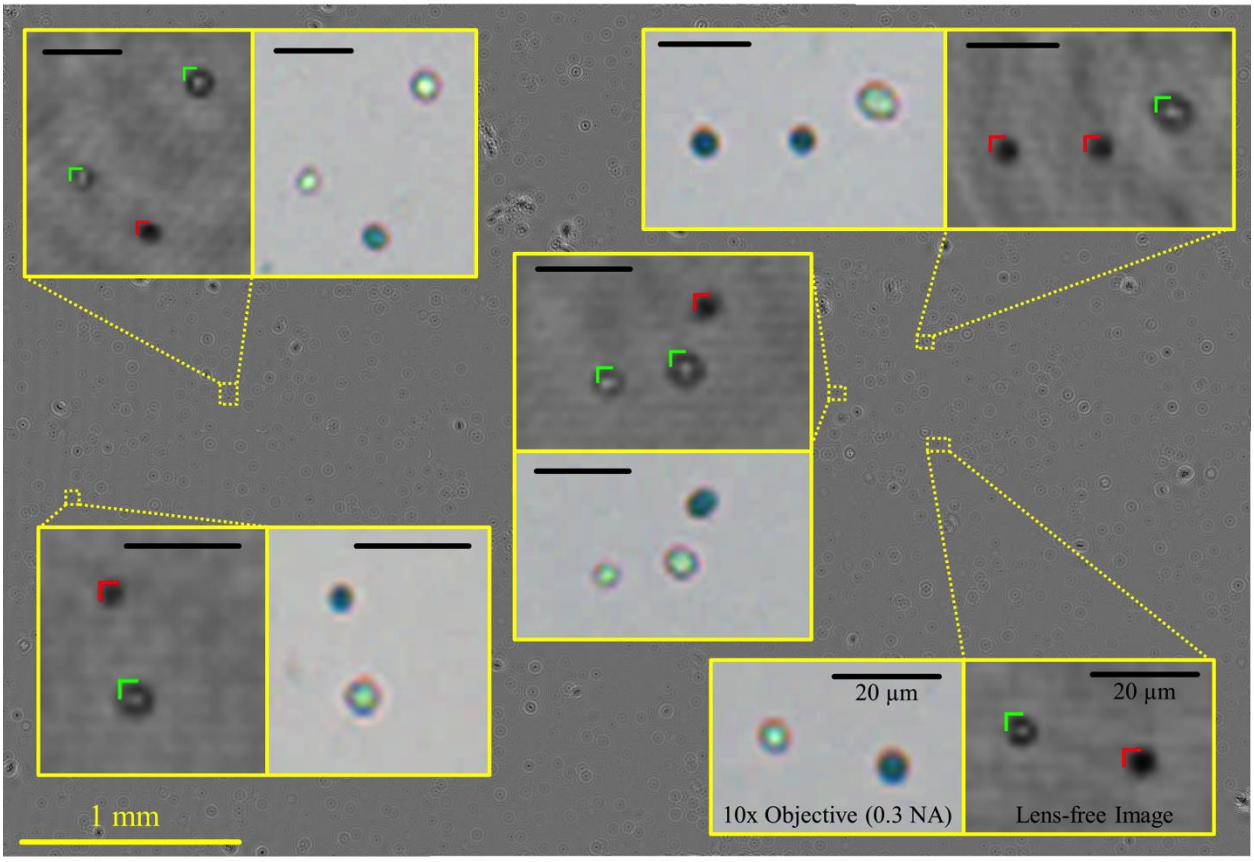


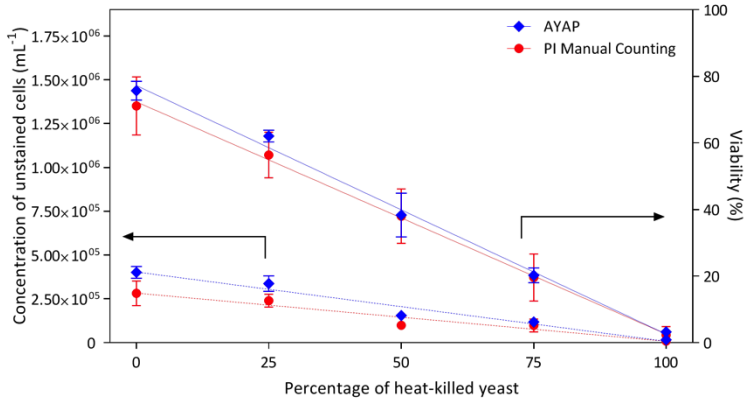
Figure 4 | The lensless microscope captures a large FOV of ~22.5 mm^2. Zoomed-in regions of the back-propagated amplitude reconstruction are displayed here. Images captured with a 10x microscope objective lens (0.3 NA) are displayed next to each zoomed-in region. Red labels indicate a stained/dead classification and the green labels indicate an unstained/viable classification by the SVM algorithm. This figure has been submitted to a peer-reviewed journal for publication [1].

samples stained with propidium iodide were captured and manually screened for stained and unstained cells.

We mixed different fractions of heat-killed yeast with the original solution in order vary the viability of our yeast samples and perform linear regression analysis. As seen in figure 5a-b, the slope and intercept for AYAP measurements agree well with fluorescence exclusion staining.

We performed serial dilution in order to test the performance of AYAP at varying concentrations, the results of which are displayed in figure 5c-d. Once again, the slope and intercept for AYAP concentration and viability measurements agree well with the gold standard

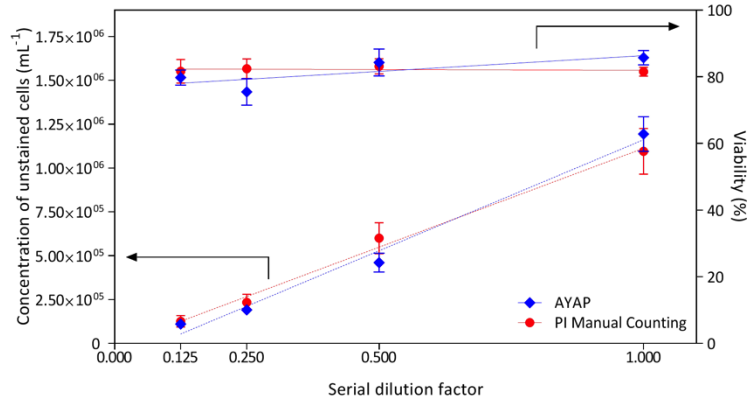
(a)



(b)

Percentage heat-killed yeast	PI manual counting		AYAP	
	# of 10x images	Total # of cells identified	# of tiles (single FOV)	Total # of cells identified
0%	5	418	6	1886
25%	5	457	6	1834
50%	4	229	6	1448
75%	5	538	6	1942
100%	5	361	6	1578
Linear regression	Slope	Y-intercept	Slope	Y-intercept
Viability	-0.6977 ± 0.01508	72.34 ± 0.9237	-0.7470 ± 0.03135	77.25 ± 1.920
Conc. of unstained cells	-2751 ± 417.1	282876 ± 25543	-3956 ± 457.4	403343 ± 28009

(c)



(d)

Serial dilution factor	PI manual counting		AYAP	
	# of 10x images	Total # of cells identified	# of tiles (single FOV)	Total # of cells identified
1.000	5	1439	6	4975
0.500	5	775	6	2081
0.250	5	304	6	878
0.125	5	166	6	518
Linear regression	Slope	Y-intercept	Slope	Y-intercept
Viability	-0.4331 ± 1.310	82.39 ± 0.7548	9.464 ± 5.238	76.90 ± 3.018
Conc. of unstained cells	1127000 ± 66950	-14190 ± 38580	1265000 ± 101100	-104000 ± 58240

(e)

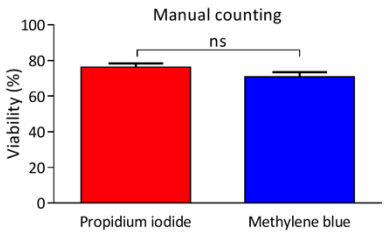


Figure 5 | Concentration and viability measured using AYAP was compared with concentration and viability measured using manual counting with propidium iodide fluorescence-based exclusion staining. (a) The viability of yeast cells was varied by mixing different fractions of heat-killed yeast with the original sample. (b) Linear regression analysis showed that the slope and y-intercept for both methods agree well with each other. (c) Concentration of yeast samples was varied by serial dilution. (d) Once again, the slope and y-intercept for both methods agree well with each other. (e) A control experiment was performed to compare manual counting using propidium iodide with manual counting with methylene. Non-parametric Mann-Whitney test showed no significant difference between viability percentages measured via either exclusion staining method. $P < 0.05$ was considered as significant difference. This figure has been submitted to a peer-reviewed journal for publication [1].

fluorescence staining method within the concentration range of 1.4×10^5 to 1.4×10^6 cells/mL. The yeast sample used in fermentation applications is typically diluted by a factor of 10 to 1,000 prior to manual counting with a hemocytometer [7]. Furthermore, conventional hemocytometers claim accurate measurement within the concentration range of 2.5×10^5 to 8×10^6 cells/mL [32]. Therefore, our portable platform's concentration range is appropriate for fermentation applications.

The small difference between AYAP's and the gold-standard method's measurement of concentration/viability may be due to variability in the microfluidic chamber height, leading to slight changes in sample volume. Another source of systematic error may be attributed to the SVM classification algorithm, which currently ignores cell clumps. During the manual counting process using fluorescence exclusion staining, we counted the number of cells within the clumps when possible.

In addition to these experiments, we performed a control experiment, which compared the viability percentages found from the manual counting of propidium iodide stained samples and methylene blue stained samples. Both of these procedures used a standard benchtop microscope to highlight the differences between the stains. In this control experiment, we separated our yeast sample into six sub-samples, three of which were stained with propidium iodide and the remaining samples were stained with methylene blue. We captured five different images from each sample using a 10x objective lens with 0.3 NA and manually counted the number of stained and unstained cells. No significant difference was observed between the viability percentages based on the Mann-Whitney test [33], as displayed in figure 5e.

4 Conclusion

We have developed a cost-effect and portable yeast cell concentration and viability measurement platform that weighs 70 g and has dimensions of 4 × 4 × 12 cm. This platform performs automatic measurement in less than 30 seconds when auto-focus in not needed. In cases where autofocus is required, this process takes 5-10 minutes using a tablet-PC. Furthermore, our platform uses the methylene blue stain, which is stable at room temperature and commercially available, making it optimal for in-field use. AYAP performs accurate viability

and concentration measurement over a large FOV of ~22.5 mm^2, allowing analysis of an order of magnitude larger number of cells compared to the number of cells visible under a microscope with a typical 10x objective lens. This large FOV is captured in less than 10 seconds, significantly reducing the number of false-positives associated with non-fluorescence exclusion staining. Moreover, SVM classification eliminates operator subjectivity in distinguishing stained and unstained cells. Comparison of our platform's performance with a fluorescence-based gold-standard method showed a dynamic concentration range of 1.4×10^5 to 1.4×10^6 cells/mL. Subsequently, the brewing and biofuel production industries would benefit from our cost-effective and portable platform.

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