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TITLE:

Analysis of Lipid Droplet Content in Fission and Budding Yeasts using Automated Image Processing

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SUMMARY:

Here, we present a MATLAB implementation of automated detection and quantitative description of lipid droplets in fluorescence microscopy images of fission and budding yeast cells.

ABSTRACT:

Lipid metabolism and its regulation are of interest to both basic and applied life sciences and biotechnology. In this regard, various yeast species are used as models in lipid metabolic research or for industrial lipid production. Lipid droplets are highly dynamic storage bodies and their cellular content represents a convenient readout of the lipid metabolic state. Fluorescence microscopy is a method of choice for quantitative analysis of cellular lipid droplets, as it relies on widely available equipment and allows analysis of individual lipid droplets. Furthermore, microscopic image analysis can be automated, greatly increasing overall analysis throughput. Here, we describe an experimental and analytical workflow for automated detection and quantitative description of individual lipid droplets in three different model yeast species: the fission yeasts *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus*, and the budding yeast *Saccharomyces cerevisiae*. Lipid droplets are visualized with BODIPY 493/503, and cell-impermeable fluorescent dextran is added to the culture media to help identify cell boundaries. Cells are subjected to 3D epifluorescence microscopy in green and blue channels and the resulting z-stack images are processed automatically by a MATLAB pipeline. The procedure outputs rich quantitative data on cellular lipid droplet

content and individual lipid droplet characteristics in a tabular format suitable for downstream analyses in major spreadsheet or statistical packages. We provide example analyses of lipid droplet content under various conditions that affect cellular lipid metabolism.

INTRODUCTION:

Lipids play crucial roles in cellular energy and carbon metabolism, synthesis of membrane components, and production of bioactive substances. Lipid metabolism is fine-tuned according to environmental conditions, nutrient availability and cell-cycle phase[1]. In humans, lipid metabolism has been connected to diseases, such as obesity, type II diabetes and cancer[2]. In industry, lipids produced by microorganisms, such as yeasts, represent a promising source of renewable diesel fuels[3]. Cells store neutral lipids in so-called lipid droplets (LDs). These evolutionarily conserved bodies are composed of triacylglycerols, steryl esters, an outer phospholipid monolayer and associated proteins[1]. LDs originate in the endoplasmic reticulum, exert cell-cycle or growth-phase dynamics, and are important for cellular lipid homeostasis[1]. LD number and morphology can be used as a convenient proxy when assaying lipid metabolism under various growth conditions or when screening a panel of mutants. Given their dynamic nature, techniques capable of analyzing the properties of individual LDs are of particular interest in studies of lipid metabolism.

Various yeast species have been used to describe lipid-related metabolic pathways and their regulation, or used in biotechnology to produce interesting compounds or fuels[1]. Furthermore, for model yeasts, such as the budding yeast *Saccharomyces cerevisiae* or the distantly related fission yeast *Schizosaccharomyces pombe*, genome-wide deletion strain libraries are available that can be used for high-throughput screens[4,5]. Recently LD composition and dynamics have been described in *S. pombe*[6–9], and mutants related to lipid metabolism have been isolated in the emerging model yeast *Schizosaccharomyces japonicus*[10].

Numerous techniques are available to study LD content and dynamics. Most employ some kind of staining of LDs with lipophilic dyes such as Nile Red or BODIPY 493/503. The latter shows more narrow excitation and emission spectra, and increased specificity towards neutral lipids (LDs) as opposed to phospholipids (membranes)[11]. Fluorimetric and flow-cytometry methods have been used successfully in various fungal species to uncover genes and growth conditions that affect storage lipid content[12–15]. While these methods are suitable for high-throughput applications, they cannot measure the numbers and morphology of individual LDs in cells, which can differ dramatically between growth conditions and genotypes. Coherent Raman scattering or digital holographic microscopy are label-free methods that yield LD-level data, but require

specialized expensive equipment[16–18]. Fluorescence microscopy, on the other hand, can provide detailed data on LD content, while utilizing commonly available instruments and image analysis software tools. Several analysis workflows exist that feature various degrees of sophistication and automation in cell/LD detection from image data, and are optimized for different cell types, such as metazoan cells with large LDs[19–21], or budding yeasts[17,22,23]. Some of these approaches only work in 2D (e.g., on maximum projection images), which may fail to reliably describe the cellular LD content. To our knowledge, no tools exist for determination of LD content and morphology from fission yeast microscopic data. Development of automated and robust LD-level analyses would bring increased sensitivity and enhanced statistical power, and provide rich information on neutral lipid content, ideally in multiple yeast species.

We have developed a workflow for LD content analysis from 3D fluorescence microscopy images of yeast cells. Live cells are stained with BODIPY 493/503 and Cascade Blue dextran to visualize LDs and determine cell boundaries, respectively. Cells are immobilized on glass slides and subjected to z-stack imaging using a standard epifluorescence microscope. Images are then processed by an automated pipeline implemented in MATLAB, a widely used (commercial) package for statistical analyses. The pipeline performs image preprocessing, segmentation (cells vs. background, removal of dead cells), and LD identification. Rich LD-level data, such as LD size and fluorescence intensity, are then provided in a tabular format compatible with major spreadsheet software tools. The workflow was used successfully to determine the impact of nitrogen source availability on lipid metabolism in *S. pombe*[24]. We now demonstrate the functionality of the workflow in *S. pombe*, *S. japonicus* and *S. cerevisiae*, using growth conditions or mutants that affect cellular LD content.

PROTOCOL:

1. Preparation of solutions and media

1.1. Prepare lipid staining solution.

1.1.1. To prepare stock lipid staining solution dissolve 10 mg of BODIPY 493/503 in 10 mL of anhydrous DMSO (final concentration 1 mg/mL). Dissolve the whole content of a 10 mg BODIPY 493/503 vial to prevent loss of material during weighing.

CAUTION: DMSO may pass through the skin. Wear appropriate personal protective equipment.

1.1.2. Prepare working lipid staining solution by mixing 100 µL of the 1 mg/mL BODIPY

493/503 stock solution and 900 µL of anhydrous DMSO (final concentration 0.1 mg/mL).

1.1.3. Aliquot the stock and working solutions, and store at -20 °C.

NOTE: Dissolved BODIPY 493/503 is stable for several years at -20 °C. However, the solution has to be protected from moisture and light.

1.2. To prepare stock solution for cell boundary visualization, dissolve 25 mg of Cascade Blue dextran (whole vial) in 2.5 mL of deionized water (final concentration 10 mg/mL). Aliquot the stock solution and store at -20 °C protected from light.

1.3. To prepare microscope slide coating solution, dissolve 5 mg of soybean lectin in 5 mL of deionized water (final concentration 1 mg/mL). Aliquot the lectin solution and store at -80 °C.

NOTE: The lectin solution is stable for several years at -80 °C. Aliquots currently at use may be stored at -20 °C.

1.4. Prepare cultivation media.

1.4.1. To prepare 400 mL of complex YES cultivation medium for *S. pombe* and *S. Japonicus,* dissolve 2 g of yeast extract and 0.1 g of SP supplements (if required for auxotrophic mutants) in 340 mL of deionized water in a 500 mL bottle and autoclave. Add 60 mL of 20% (w/v) of separately autoclaved or filter-sterilized glucose in aseptic conditions.

1.4.2. To prepare 400 mL of defined EMM cultivation medium for *S. pombe* and *S. Japonicus,* dissolve 4.9 g of EMM broth without dextrose in 360 mL of deionized water in a 500 mL bottle and autoclave. Add 40 mL of 20% (w/v) of separately autoclaved or filter-sterilized glucose in aseptic conditions.

NOTE: For general guidelines on *S. pombe* and *S. japonicus* cultivation see[25] and[26], respectively.

1.4.3. To prepare 300 mL of complex YPAD cultivation medium for *Saccharomyces* cerevisiae, dissolve 3 g of yeast extract, 6 g of peptone and 30 mg of adenine sulphate in 270 mL of deionized water in a 500 mL bottle and autoclave. Add 30 mL of 20% (w/v) of separately autoclaved or filter-sterilized glucose in aseptic conditions.

1.4.4. To prepare 300 mL of defined minimal medium for *S. Cerevisiae,* dissolve 2 g of

yeast nitrogen base (without amino acids) in 270 mL of deionized water in a 500 mL bottle and autoclave. Add 30 mL of 20% (w/v) of separately autoclaved or filter-sterilized glucose in aseptic conditions.

NOTE: For general guidelines on *S. cerevisiae* cultivation see[27].

2. Cell cultivation

2.1. Growing *S. pombe* or *S. japonicus* to exponential or early stationary phase.

2.1.1. In the morning, inoculate 5 mL of YES medium with fresh fission yeast biomass. Incubate at 32 °C with shaking (180 rpm) for several hours.

NOTE: For all cultivations, use Erlenmeyer flasks having 10 times the volume of culture to ensure proper aeration. Some laboratories prefer to grow fission yeasts at 30 °C, but cultivation temperature of 32 °C results in shorter doubling times without detrimental effects to the cells, thus reducing the total time required to perform an experiment[25,28].

2.1.2. In late afternoon of the same day (after at least 6 hours of cultivation), dilute the culture with fresh YES medium to a 10 mL final culture volume so that it reaches the desired optical density (OD) (or number of cells/mL) the following morning, and incubate at 32 °C with shaking (180 rpm). It is of advantage to know the doubling time of each used strain to accurately determine the dilution factor (use Equation 1).

Equation 1 $$V_{culture} = \frac{V_{final} \cdot OD_{final}}{OD_{current} \cdot 2^{\frac{t - t_{lag}}{t_{DT}}}}$$

Where $V_{culture}$ is the preculture volume needed for dilution, V_{final} is the total volume of the new culture (10 mL for standard cultivations), OD_{final} is the desired OD to be reached the following morning, $OD_{current}$ is the currently measured OD of the preculture, t is the time of cell growth until harvesting, t_{lag} is duration of the lag phase (depends on laboratory conditions, needs to be empirically defined) and t_{DT} is the doubling time of the strain.

NOTE: When exponential-phase cells are to be analyzed, do not let precultures reach the stationary phase as this dramatically alters cell physiology (including LD content) for several subsequent generations.

2.1.3. In the morning of imaging day, if the culture reached slightly higher OD than required (in case of exponential-phase cells), dilute it with fresh YES and continue

incubation for at least two more doubling times before staining of LDs. Otherwise proceed directly to staining (Section 3).

2.2. Growing *S. cerevisiae* to exponential and stationary phase.

2.2.1. In the afternoon, inoculate 10 mL of YPAD medium with a small amount of fresh budding yeast biomass and incubate overnight at 30 °C with shaking (180 rpm).

2.2.2. The morning of imaging day, dilute the culture to OD 0.1 in 10 mL of YPAD medium and grow to the required OD (e.g., OD 1 for exponential phase). Perform any culture dilutions as described in step 2.1.2. Proceed to staining (Section 3).

3. Lipid droplet staining

3.1. Prepare a microscope cover slip for each sample to be imaged. Spread 1 µL of slide coating solution onto a clean cover slip using the long side of a horizontally positioned pipette tip. Allow the coating solution to dry completely and store the cover slips in a dust-free environment.

NOTE: Glass slides and coverslips can be cleaned prior to use if required. The cleaning procedure consists of washing with dishwashing detergent, rinsing with water, overnight soaking in 3% hydrochloric acid, and washing with distilled water. Cleaned slides and coverslips are stored in pure ethanol until use.

3.2. Measure the OD of cell culture or number of cells/mL, as required. Try to reach similar values among all tested strains to ensure comparable experimental conditions.

3.3. Pipette 1 mL of each cell culture to a 1.5 mL microcentrifuge tube. For *S. cerevisiae* only, add 5 µL of the slide coating solution, vortex briefly, and incubate at 30 °C with shaking for 5 min.

3.4. Add 1 µL of the lipid staining solution to each culture aliquot and vortex briefly. Then add 10 µL of the cell boundary visualization solution and vortex briefly.

NOTE: Do not prepare pre-mixed solutions of both stains as this leads to fluorescence quenching of BODIPY 493/503.

3.5. Collect the cells by centrifugation (1,000 x *g*, 3 min, RT) and remove almost all supernatant (~950 µL). Resuspend the cells in the remaining supernatant.

3.6. Pipette 2 µL of the dense cell suspension on a lectin-coated cover slip and place onto a clean microscope slide. The cells should form a monolayer. Proceed to microscopy (Section 4) as quickly as possible to minimize artefacts in imaging; process maximum of two samples at a time.

4. Setting up the microscope and imaging

4.1. Optimize imaging conditions.

NOTE: Setting up the microscope requires long exposures to strong light sources that could cause damage to the sample and skew results. Therefore, set up the imaging conditions using a dedicated sample slide that will not be further used for LD quantification.

4.1.1. Focus on the cells using phase contrast or differential interference contrast (DIC).

NOTE: Phase contrast or DIC images may be taken for reference, but they are not used during the automated image analysis step.

4.1.2. Set Z-stack settings to span the whole cell volume. The total vertical distance depends on the cell size; the number of optical slices depends on the numerical aperture of the objective (point spread function in z-axis). Set the focus to move relative to the central focal plane.

NOTE: The optimal number of slices is often set by the microscope control software and does not need to be calculated manually. The typical cell widths are 3-5 µm for S. pombe, 4-7 µm for S. japonicus, and 3-7 µm for S. cerevisiae.

4.1.3. To image LDs, set light intensity and exposure time in the green channel (excitation and emission maxima of BODIPY are 493 and 503 nm, respectively).

NOTE: BODIPY 493/503 is a very bright fluorochrome; however, it may get bleached rapidly with overly strong light intensity. Moreover, LDs are mobile in live cells, thus minimize exposure time and capture the full green-channel z-stack first (before switching to the blue channel) to prevent blurring artifacts. Also, take into account the linear range of the camera for signal intensity to avoid saturated pixels.

4.1.4. To image cell boundaries, set light intensity and exposure time in the blue channel (excitation and emission maxima of Cascade Blue dextran are 400 and 420 nm, respectively).

NOTE: Signal intensity in the blue channel is required for image segmentation, but it is not used for LD quantification itself. Therefore, optimal settings in this channel are not crucial for analysis.

4.1.5. If possible, create an automated experimental workflow in the microscope control software to facilitate imaging of multiple samples under standardized conditions.

4.2. Once imaging conditions have been optimized, image samples to be used for quantification. Focus on the cells and image them in green and blue channels as described in Step 4.1.

NOTE: All images must be acquired using the same settings to allow comparison between samples. Image multiple fields of view per sample to obtain robust, representative data.

4.3. Save the blue and green channel Z-stack images as 16-bit multi-layer TIFF files (i.e., two files per field of view). Include words “green” or “blue” in the corresponding file names. Proceed with image analysis (Section 5).

5. Image analysis

5.1. Visually check the quality of acquired images.

5.1.1. Open microscopic images in ImageJ[29,30] or other suitable image analysis software.

5.1.2. Remove any image stacks containing a considerable number of cells that moved during acquisition (and thus created blurring artifacts).

5.1.3. Remove any image stacks containing highly fluorescent non-cell particles in the blue channel (e.g., dirt on microscope slide or cover slip, impurities in cultivaton medium).

NOTE: Very bright non-cell objects in the blue channel may create cell detection artifacts or may interfere with detection of cells in their vicinity.

5.1.4. Remove any image stacks containing a large proportion of dead cells (i.e., cells with increased blue fluorescence compared to live cells).

NOTE: While the presence of a small proportion of dead cells in the sample is typically not a problem and these cells are automatically discarded during analysis, some dead or dying cells may occasionally be recognized as live cells by the segmentation algorithm and thus skew the reported results.

5.2. Analyze images in the MATLAB software.

5.2.1. Create a main folder and copy all MATLAB scripts to this location.

5.2.2. Create a sub-folder (“pombe”, “cerevisiae” or “japonicus”) and copy input TIFF image files to this location.

5.2.3. Start MATLAB, open script MAIN.m and run it. In the menu select the yeast species to be analyzed and start image processing.

NOTE: Some of the parameters required for cell and LD detection are pre-set for the particular species, others are determined automatically during image processing. The pre-set values were determined empirically and depend on several factors such as objective magnification, camera type and sensitivity, and imaging settings. If required, users may edit the script files to change the organism-specific presets to better reflect their experimental setup. Namely, during cell recognition acceptable object sizes are given by the “minArea” and “maxArea” parameters, and the minimum fraction of filled volume within the object boundaries is given by the “Solidity” parameter. For LD recognition, the brightness threshold is given by the “th” parameter (its value is affected mostly by image bit depth and fluorescence signal intensity), and maximum acceptable LD size is given by the “MaxArea” parameter.

5.2.4. Inspect and process the output files as required using a spreadsheet editor or statistical package; the workflow produces semicolon-separated CSV files, and segmented TIFF files with detected cell objects and LDs.

NOTE: The workflow segments images into background and cell objects, where each cell object may be composed of multiple adjacent cells. Therefore, the output in “xxxx_cells.csv” files does not represent single-cell data and should only be used to calculate per-unit-of-cell-volume metrics.

REPRESENTATIVE RESULTS:

The whole procedure is summarized in **Figure 1** for the fission yeasts (the budding yeast workflow is analogous), and below we provide examples of how the workflow can be used to study LD content in three different yeast species under various conditions

known to affect cellular LD content. Each example represents a single biological experiment.

[Place Figure 1 here]

First, we analyzed *S. pombe* cells (**Figure 2**). Wild-type (WT; h^{+s}) cells were grown to exponential phase in either the complex YES medium or defined EMM medium. Compared to YES, fewer LDs and higher LD staining intensity per unit of cell volume were detected in EMM (**Figure 2A-C**). Moreover, individual LDs formed in EMM medium were larger and displayed increased total staining intensity (**Figure 2D, E**). This is in agreement with previous findings of increased storage lipid content in cells grown in EMM[24]. The *ppc1* gene encodes a phosphopantothenate-cysteine ligase required for coenzyme A synthesis. The temperature-sensitive *ppc1-88* mutant shows a marked decrease in LD content when grown at the restrictive temperature[31], providing an example of cells with low BODIPY 493/503 signal (**Figure 2A**). Accordingly, compared to wild type (grown at 32°C), smaller LDs with lower total staining intensity were detected in *ppc1-88* cells grown in YES following a shift to 36°C (**Figure 2D, E**), without any apparent change in LD number per unit of cell volume (**Figure 2B**).

[Place Figure 2 here]

Next, we quantified LD content in *S. japonicus* cells (*h⁺ matsj-2017*)[32] from exponential and early-stationary cultures grown in YES (**Figure 3A**). Cells entering stationary phase showed markedly decreased number of LDs per unit of cell volume compared to exponentially growing cells (**Figure 3B**), while volume-normalized LD fluorescence intensity decreased slightly between the two conditions (**Figure 3C**). The early stationary-phase LDs were typically moderately larger in size and had moderately higher total fluorescence intensity compared to LDs from exponentially growing cells (**Figure 3D, E**).

[Place Figure 3 here]

Finally, we analyzed *S. cerevisiae* cells of the widely used BY4741 laboratory strain (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) grown to exponential and stationary phase, respectively, in the complex YPAD medium. Budding yeast cells typically accumulate storage lipids upon entry into stationary phase[1], and we were able to recapitulate these findings (**Figure 4**). Stationary cells contained somewhat fewer LDs per unit of volume compared to exponentially growing cells (**Figure 4B**), but their volume-normalized LD fluorescence intensity almost doubled (**Figure 4C**). This sharp increase in overall LD content was due to the much higher fluorescence intensity and volume of individual LDs

in stationary phase (**Figure 4D, E**).

[Place Figure 4 here]

Thus, our analysis workflow can detect changes in LD number, size and lipid content in three different and morphologically distinct yeast species under various conditions that positively or negatively affect cellular LD content.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the experimental and analytical workflow. The workflow for fission yeasts is shown as an example.

Figure 2: Impact of growth media and lipid metabolism mutation on LD content in *S. pombe*. Wild type (WT) and *ppc1-88* cells were grown to exponential phase in the complex YES or defined EMM medium, as indicated. WT cells were grown at 32°C. The temperature-sensitive *ppc1-88* cells were grown at 25°C and shifted to 36°C for 2 hours prior to analysis. (**A**) Representative unprocessed microscopic images of LDs stained with BODIPY 493/503. A single optical slice is shown for each condition; 10% overlay with inverted blue channel was added to better visualize cell boundaries. Scale bar represents 10 µm. (**B**) Number of identified LDs per unit of cell volume. (**C**) Fluorescence intensity of identified LDs per unit of cell volume. (**D**) Distributions of total fluorescence intensities of all identified LDs. ***, ### unpaired Wilcoxon test $p = 1.7 \times 10^{-107}$, $p = 3.7 \times 10^{-132}$, respectively. (**E**) Distributions of volumes of all identified LDs. ***, ### unpaired Wilcoxon test $p = 6.8 \times 10^{-71}$, $p = 1 \times 10^{-64}$, respectively. Data in panels B-E were derived from 242, 124 and 191 cell objects for the WT YES, WT EMM and *ppc1-88* samples, respectively.

Figure 3: LD content in *S. japonicus* cells changes with growth phase. Exponentially growing (LOG) and early stationary phase (STAT) cells were analyzed. (**A**) Representative unprocessed microscopic images of LDs stained with BODIPY 493/503. A single optical slice is shown for each condition; 10% overlay with inverted blue channel was added to better visualize cell boundaries. Scale bar represents 10 µm. (**B**) Number of identified LDs per unit of cell volume. (**C**) Fluorescence intensity of identified LDs per unit of cell volume. (**D**) Distributions of total fluorescence intensities of all identified LDs. *** unpaired Wilcoxon test $p = 1.3 \times 10^{-114}$. (**E**) Distributions of volumes of all identified LDs. *** unpaired Wilcoxon test $p = 2.4 \times 10^{-85}$. Data in panels B-E were derived from 274 and 187 cell objects for the LOG and STAT samples, respectively.

Figure 4: LD content in *S. cerevisiae* cells changes with growth phase.

Exponentially growing (LOG) and stationary phase (STAT) cells were analyzed. (**A**) Representative unprocessed microscopic images of LDs stained with BODIPY 493/503. A single optical slice is shown for each condition; 10% overlay with inverted blue channel was added to better visualize cell boundaries. Scale bar represents 10 µm. (**B**) Number of identified LDs per unit of cell volume. (**C**) Fluorescence intensity of identified LDs per unit of cell volume. (**D**) Distributions of total fluorescence intensities of all identified LDs. *** unpaired Wilcoxon test $p = 4.6 \times 10^{-78}$. (**E**) Distributions of volumes of all identified LDs. *** unpaired Wilcoxon test $p = 3.7 \times 10^{-63}$. Data in panels B-E were derived from 430 and 441 cell objects for the LOG and STAT samples, respectively.

DISCUSSION:

The understanding of lipid metabolism and its regulation is important for both basic biology, and clinical and biotechnological applications. LD content represents a convenient readout of lipid metabolism state of the cell, with fluorescence microscopy being one of the major methods used for LD content determination. The presented protocol allows automated detection and quantitative description of individual LDs in three different and morphologically distinct yeast species. To our knowledge, no similar tools exist for the fission yeasts. The MATLAB scripts required for image processing are included as Supplementary files, and are also available from the Figshare repository (DOI 10.6084/m9.figshare.7745738) together with all raw and processed image and tabular data from this manuscript, detailed descriptions of the CSV output files, and R scripts for downstream data analysis and visualization. Also, the latest version of the MATLAB scripts is available from GitHub (https://github.com/MartinSchatzCZ/LipidDots-analysis).

Successful LD analysis is largely dependent on the quality of the raw fluorescence images obtained. For optimal performance of the segmentation algorithms, clean glass slides devoid of dust particles should be used for microscopy, the cells should form a monolayer (the actual number of cells per field of view is not a critical parameter), and should not contain a large proportion of dead cells. Also, the Z-stack imaging should start slightly below and end slightly above the cells. Depending on the particular microscopic setup, users may need to adjust some of the parameters in the image processing scripts (such as “th” for image background intensity threshold). While the current method is able to detect and describe individual LDs in the segmented cell objects, the workflow does not produce truly single-cell data due to difficulties with automated separation of all individual cells. Instead, LD content per unit of cell volume generalized for the whole sample is reported. This limitation may hamper data interpretation in analyses of heterogeneous cell populations. Also, care should be taken when working with cells with altered transport of small molecules (e.g., efflux pump mutants), as this might affect the intracellular BODIPY 493/503 concentration and LD

staining, as observed for the Nile Red lipophilic dye[33,34].

Staining the medium with the cell-impermeable Cascade Blue fluorescent dextran is a convenient way of distinguishing cells from the background[35], which can be applied to many (if not all) yeast species. It also helps with automated removal of dead cells from the analysis as these will turn blue upon staining. Any dying or sick (and thus partially permeable for dextran) cells detected as alive can be removed during data analysis steps based on the "IntensityMedianBlue" value of the detected cell objects. In principle, the whole workflow can be used to detect various other cellular structures, such as DNA repair foci, provided the structures can be labelled with suitable fluorophores. The workflow should also be applicable to cells of other (yeast) species, further broadening its utility.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Koch, B., Schmidt, C., Daum, G. Storage lipids of yeasts: a survey of nonpolar lipid metabolism in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*. *FEMS Microbiology Reviews*. **38** (5), 892–915, doi: 10.1111/1574-6976.12069 (2014).

2. Krahmer, N., Farese, R. V., Walther, T.C. Balancing the fat: lipid droplets and human disease. *EMBO Molecular Medicine*. **5** (7), 973–83, doi: 10.1002/emmm.201100671 (2013).

3. Lazar, Z., Liu, N., Stephanopoulos, G. Holistic Approaches in Lipid Production by *Yarrowia lipolytica*. *Trends in Biotechnology*. **36** (11), 1157–1170, doi: 10.1016/j.tibtech.2018.06.007 (2018).

4. Kim, D.-U. et al. Analysis of a genome-wide set of gene deletions in the fission yeast *Schizosaccharomyces pombe*. *Nature Biotechnology*. **28** (6), nbt.1628-9, doi: 10.1038/nbt.1628 (2010).

5. Giaever, G., Nislow, C. The yeast deletion collection: a decade of functional genomics. *Genetics*. **197** (2), 451–65, doi: 10.1534/genetics.114.161620 (2014).

6. Meyers, A. et al. The protein and neutral lipid composition of lipid droplets isolated from the fission yeast, *Schizosaccharomyces pombe*. *Journal of Microbiology (Seoul, Korea)*. **55** (2), 112–122, doi: 10.1007/s12275-017-6205-1 (2017).

7. Meyers, A. et al. Lipid Droplets Form from Distinct Regions of the Cell in the Fission Yeast *Schizosaccharomyces pombe*. *Traffic (Copenhagen, Denmark)*. **17** (6), 657–69, doi: 10.1111/tra.12394 (2016).

8. Long, A.P. et al. Lipid droplet de novo formation and fission are linked to the cell cycle in fission yeast. *Traffic (Copenhagen, Denmark)*. **13** (5), 705–14, doi: 10.1111/j.1600-0854.2012.01339.x (2012).

9. Yang, H.-J., Osakada, H., Kojidani, T., Haraguchi, T., Hiraoka, Y. Lipid droplet dynamics during *Schizosaccharomyces pombe* sporulation and their role in spore survival. *Biology Open*. 8–10, doi: 10.1242/bio.022384 (2016).

10. Aoki, K., Shiwa, Y., Takada, H., Yoshikawa, H., Niki, H. Regulation of nuclear envelope dynamics via APC/C is necessary for the progression of semi-open mitosis in *Schizosaccharomyces japonicus*. *Genes To Cells: Devoted To Molecular & Cellular Mechanisms*. **18** (9), 733–52, doi: 10.1111/gtc.12072 (2013).

11. Karolin, J., Johansson, L.B.A., Strandberg, L., Ny, T. Fluorescence and Absorption Spectroscopic Properties of Dipyrrometheneboron Difluoride (BODIPY) Derivatives in Liquids, Lipid Membranes, and Proteins. *Journal of the American Chemical Society*. **116** (17), 7801–7806, doi: 10.1021/ja00096a042 (1994).

12. Bozaquel-Morais, B.L., Madeira, J.B., Maya-Monteiro, C.M., Masuda, C.A., Montero-Lomeli, M. A new fluorescence-based method identifies protein phosphatases regulating lipid droplet metabolism. *PloS One*. **5** (10), e13692, doi: 10.1371/journal.pone.0013692 (2010).

13. Sitepu, I.R. et al. An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *Journal of Microbiological Methods*. **91** (2), 321–8, doi: 10.1016/j.mimet.2012.09.001 (2012).

14. Rostron, K.A., Lawrence, C.L. Nile Red Staining of Neutral Lipids in Yeast. *Methods in Molecular Biology (Clifton, N.J.)*. **1560**, 219–229, doi: 10.1007/978-1-4939-6788-9_16 (2017).

15. Romero-Aguilar, L., Montero-Lomeli, M., Pardo, J.P., Guerra-Sánchez, G. Lipid Index Determination by Liquid Fluorescence Recovery in the Fungal Pathogen *Ustilago Maydis*. *Journal of Visualized Experiments*. (134), 1–6, doi: 10.3791/57279 (2018).

16. Gupta, A., Dorlhiac, G.F., Streets, A.M. Quantitative imaging of lipid droplets in single cells. *The Analyst*. doi: 10.1039/c8an01525b (2018).

17. Wolinski, H., Bredies, K., Kohlwein, S.D. Quantitative imaging of lipid metabolism in yeast: from 4D analysis to high content screens of mutant libraries. *Methods in Cell Biology*. **108**, 345–65, doi: 10.1016/B978-0-12-386487-1.00016-X (2012).

18. Campos, V., Rappaz, B., Kuttler, F., Turcatti, G., Naveiras, O. High-throughput, nonperturbing quantification of lipid droplets with digital holographic microscopy. *Journal of Lipid Research*. **59** (7), 1301–1310, doi: 10.1194/jlr.D085217 (2018).

19. Ranall, M. V., Gabrielli, B.G., Gonda, T.J. High-content imaging of neutral lipid droplets with 1,6-diphenylhexatriene. *BioTechniques*. **51** (1), 35–6, 38–42, doi: 10.2144/000113702 (2011).

20. Schnitzler, J.G. et al. Nile Red Quantifier: a novel and quantitative tool to study lipid accumulation in patient-derived circulating monocytes using confocal microscopy. *Journal of Lipid Research*. **58** (11), 2210–2219, doi: 10.1194/jlr.D073197 (2017).

21. Bombrun, M., Gao, H., Ranefall, P., Mejhert, N., Arner, P., Wählby, C. Quantitative high-content/high-throughput microscopy analysis of lipid droplets in subject-specific adipogenesis models. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*. **91** (11), 1068–1077, doi: 10.1002/cyto.a.23265 (2017).

22. Capus, A., Monnerat, M., Ribeiro, L.C., de Souza, W., Martins, J.L., Sant'Anna, C. Application of high-content image analysis for quantitatively estimating lipid accumulation in oleaginous yeasts with potential for use in biodiesel production. *Bioresource Technology*. **203**, 309–17, doi: 10.1016/j.biortech.2015.12.067 (2016).

23. Lv, X. et al. Identification of gene products that control lipid droplet size in yeast using a high-throughput quantitative image analysis. *Biochimica et biophysica acta. Molecular and Cell Biology Of Lipids*. **1864** (2), 113–127, doi: 10.1016/j.bbalip.2018.11.001 (2018).

24. Zach, R., Tvarůžková, J., Schätz, M., Ťupa, O., Grallert, B., Převorovský, M. Mitotic defects in fission yeast lipid metabolism "cut" mutants are suppressed by ammonium chloride. *FEMS Yeast Research*. **18** (6), 1–7, doi: 10.1093/femsyr/foy064 (2018).

25. Petersen, J., Russell, P. Growth and the Environment of *Schizosaccharomyces pombe*. *Cold Spring Harbor Protocols*. **2016** (3), pdb.top079764, doi: 10.1101/pdb.top079764 (2016).

26. Aoki, K., Furuya, K., Niki, H. *Schizosaccharomyces japonicus*: A Distinct Dimorphic Yeast among the Fission Yeasts. *Cold Spring Harbor Protocols*. **2017** (12),

pdb.top082651, doi: 10.1101/pdb.top082651 (2017).

27. Curran, B.P.G., Bugeja, V. Basic investigations in Saccharomyces cerevisiae. *Methods in Molecular Biology (Clifton, N.J.)*. **1163**, 1–14, doi: 10.1007/978-1-4939-0799-1_1 (2014).

28. Sabatinos, S.A., Forsburg, S.L. Molecular genetics of *Schizosaccharomyces pombe*. *Methods in Enzymology*. **470** (10), 759–95, doi: 10.1016/S0076-6879(10)70032-X (2010).

29. Schindelin, J., Rueden, C.T., Hiner, M.C., Eliceiri, K.W. The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular Reproduction and Development*. **82** (7–8), 518–29, doi: 10.1002/mrd.22489 (2015).

30. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nature Methods*. **9** (7), 676–82, doi: 10.1038/nmeth.2019 (2012).

31. Nakamura, T., Pluskal, T., Nakaseko, Y., Yanagida, M. Impaired coenzyme A synthesis in fission yeast causes defective mitosis, quiescence-exit failure, histone hypoacetylation and fragile DNA. *Open Biology*. **2** (9), 120117, doi: 10.1098/rsob.120117 (2012).

32. Furuya, K., Niki, H. Isolation of heterothallic haploid and auxotrophic mutants of *Schizosaccharomyces japonicus*. *Yeast*. **26** (4), 221–233, doi: 10.1002/yea.1662 (2009).

33. Ivnitski-Steele, I. et al. Identification of Nile red as a fluorescent substrate of the *Candida albicans* ATP-binding cassette transporters Cdr1p and Cdr2p and the major facilitator superfamily transporter Mdr1p. *Analytical Biochemistry*. **394** (1), 87–91, doi: 10.1016/j.ab.2009.07.001 (2009).

34. Wolinski, H., Kohlwein, S.D. Microscopic analysis of lipid droplet metabolism and dynamics in yeast. *Methods in Molecular Biology (Clifton, N.J.)*. **457** (1), 151–63, doi: 10.1007/978-1-59745-261-8 (2008).

35. Graml, V. et al. A genomic Multiprocess survey of machineries that control and link cell shape, microtubule organization, and cell-cycle progression. *Developmental Cell*. **31** (2), 227–239, doi: 10.1016/j.devcel.2014.09.005 (2014).

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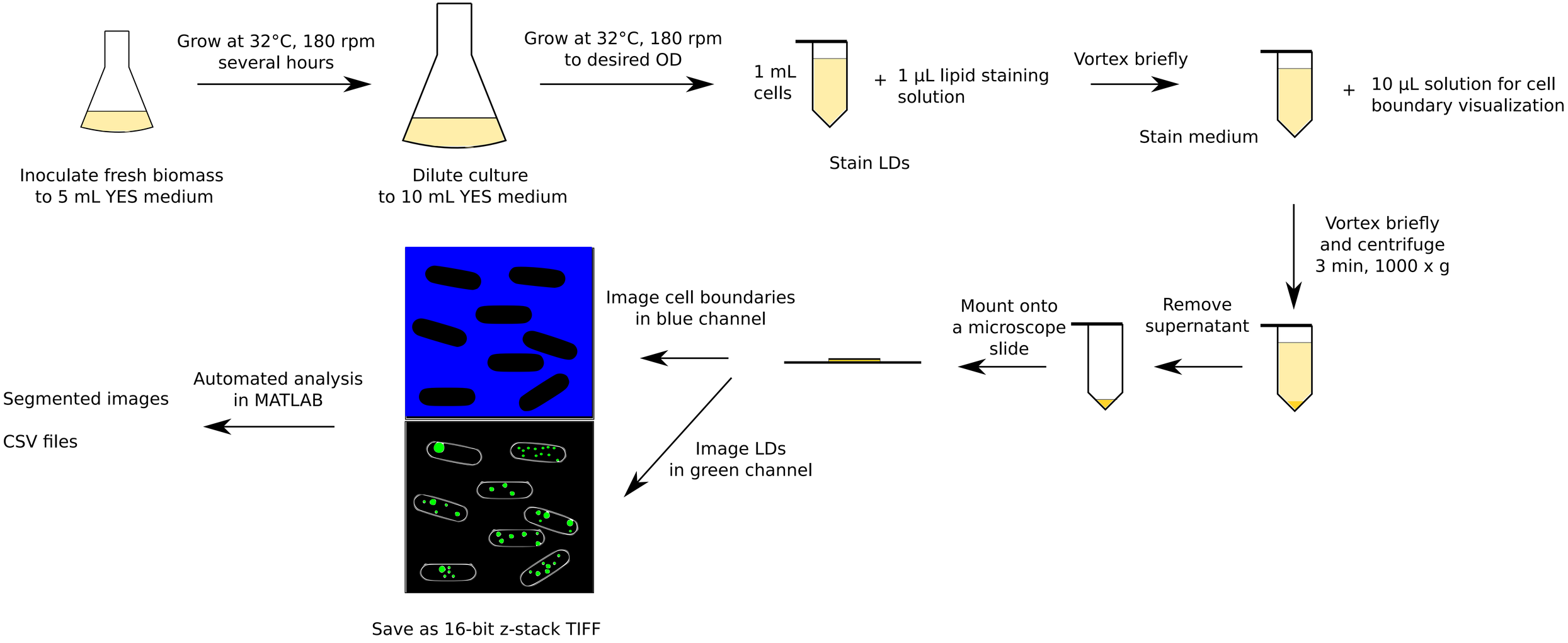
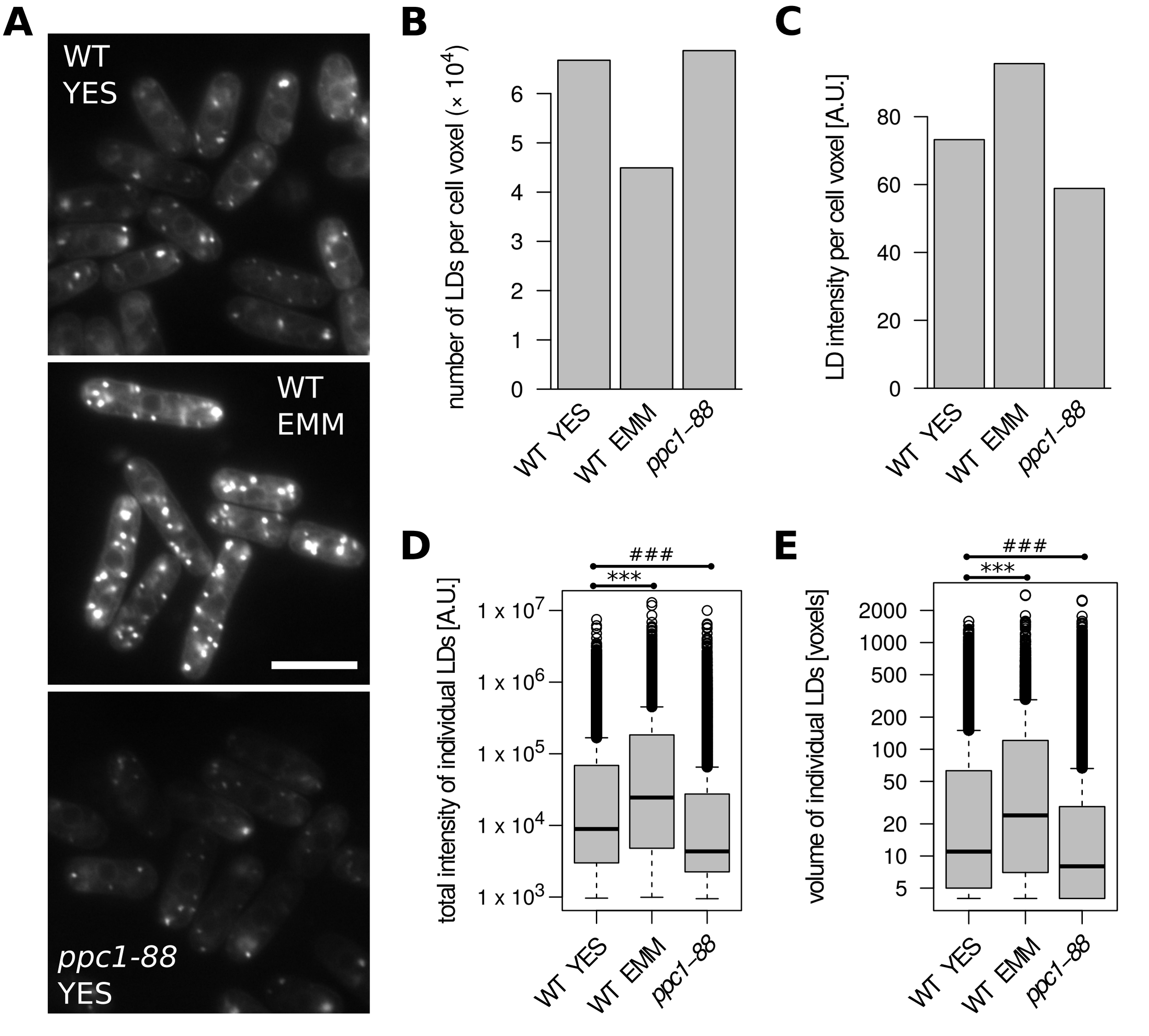


Figure 2



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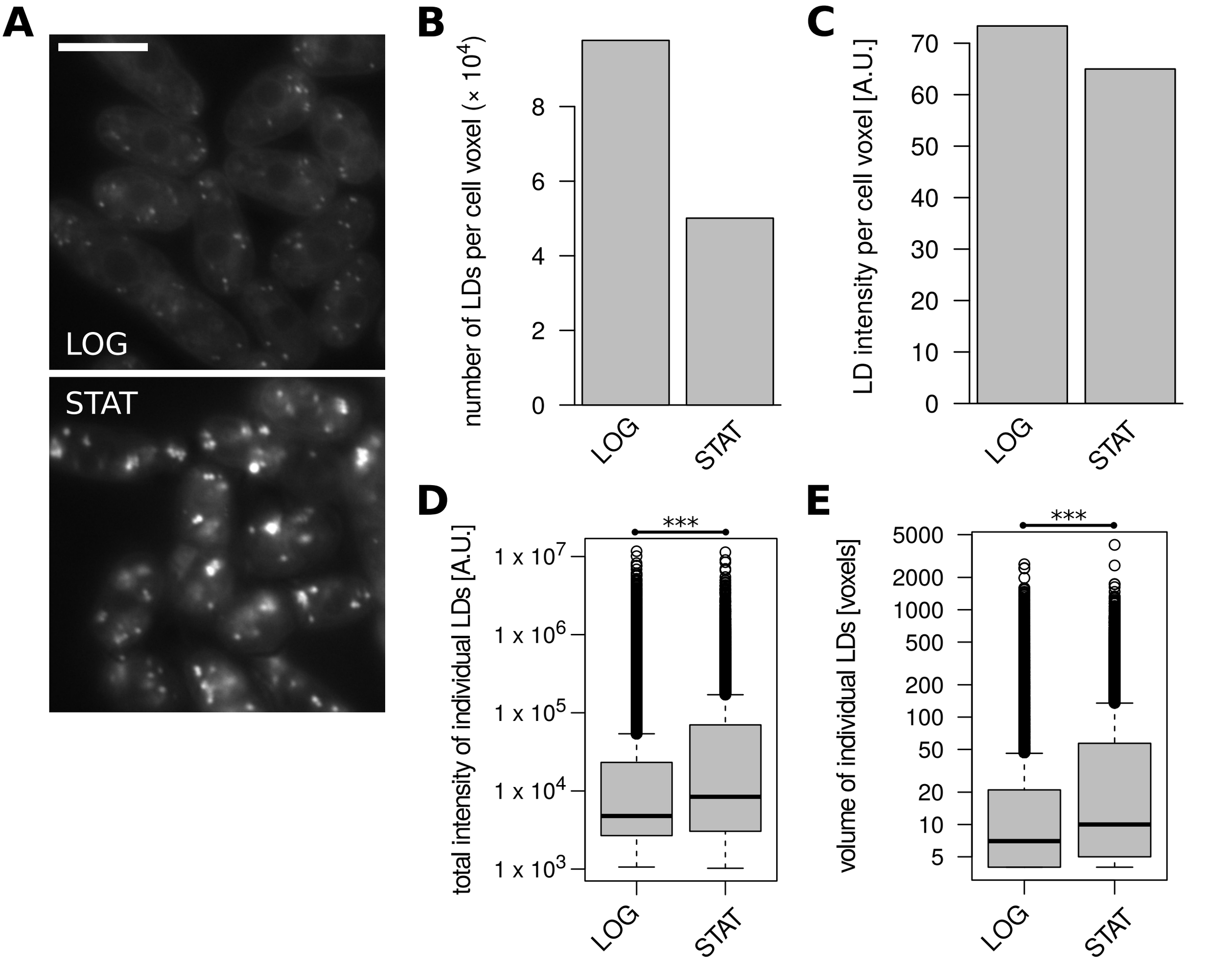
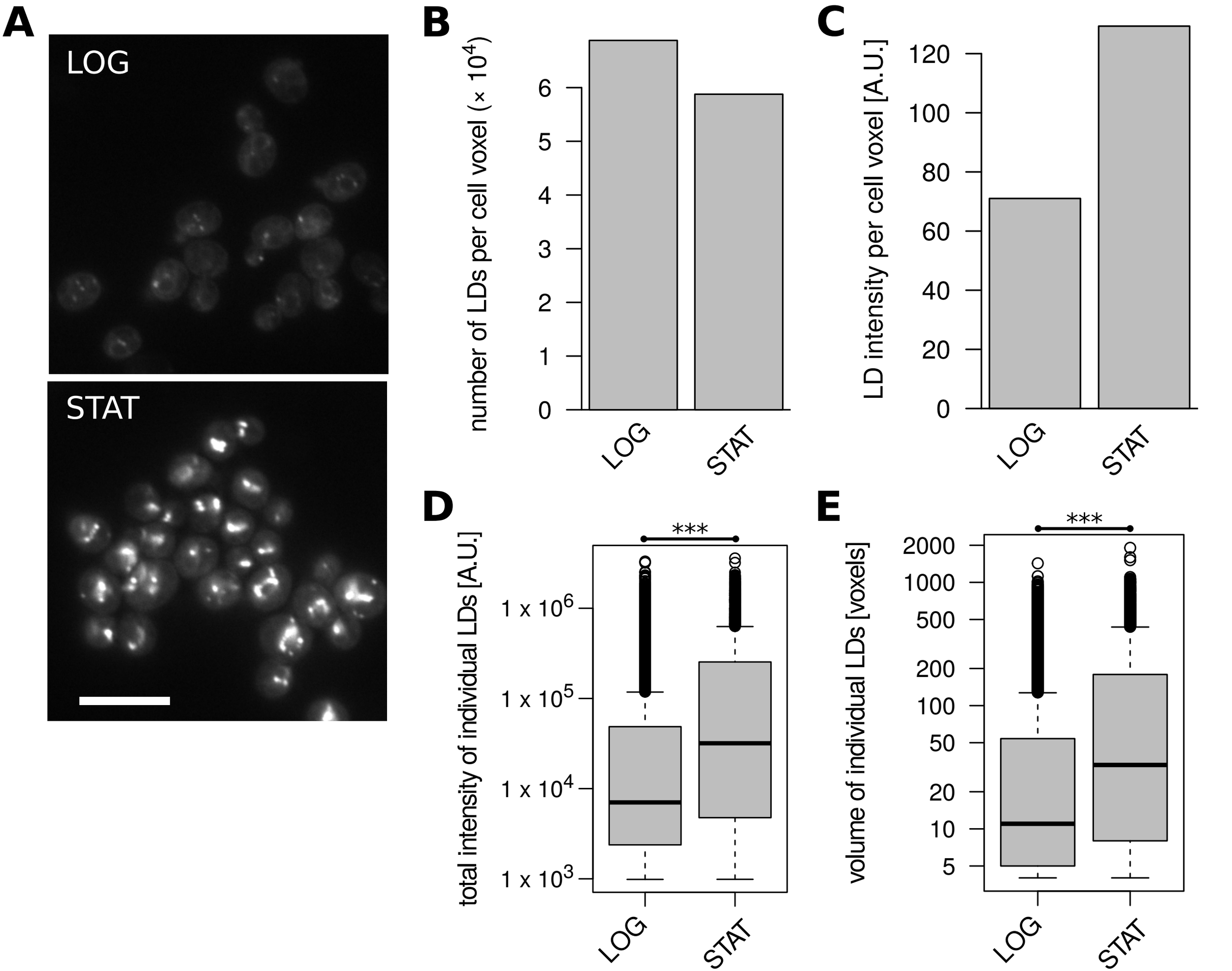


Figure 4



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BODIPY 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indac	Thermo Fisher Scientifi
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Fiji/ImageJ software	NIH
High precision cover glasses, 22x22 mm, No 1.5	VWR
Image Processing Toolbox for MATLAB, version 10.0	Mathworks
Lectin from Glycine max (soybean)	Merck
MATLAB software, version 9.2	Mathworks
Microscope slide, 26 x 76 mm, 1 mm thickness	Knittel Glass
Olympus CellR microscope with automatic z-axis objective movement	Olympus
pentaband filter set	Semrock
Signal Processing Toolbox for MATLAB, version 7.4	Mathworks
SP supplements	Formedium
standard office computer capable of running MATLAB	
Statistics and Machine Learning Toolbox for MATLAB, version 11.1	Mathworks
Universal peptone M66 for microbiology	Merck
UPLSAPO 60XO objective	Olympus
Yeast extract	Formedium
Yeast nitrogen base without amino acids	Formedium

Catalog Number	Comments/Description
	or equivalent
A9126-25G	
D3922	for neutral lipid staining
G7021	
D1976	for negative staining of cells
D4540	or higher purity, keep anhydrous on molecular sieves
PMD0405	medium may also be prepared from individual components
	or equivalent; for visual inspection of microscopic data
630-2186	use any # 1.5 cover glass
L1395	for cell immobilization on slides
L762601.2	use any microscope slide fitting your microscope stage, clean thoroughly b
	or equivalent
F66-985	brightfield, green and blue channels are sufficient
PSU0101	
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	or equivalent
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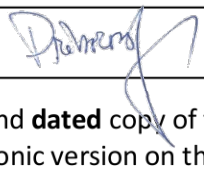
Signature:	
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3. Figure 1: Please capitalize the L in the microliter and milliliter abbreviation: mL, etc. Please remove the term Matlab.

Volume units fixed.

Ad MATLAB, please see our response to point 6 below

4. Figure 2B/3B/4B: Please superscript the 4 to be an exponent instead of using ^4.

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For example: Matlab, etc.

The analysis scripts that are integral to our workflow are written in the MATLAB scripting language, use heavily MATLAB image analysis libraries, and cannot be used in any other statistical package. Therefore, the (commercial) MATLAB package, which is often used in scientific settings, needs to be explicitly mentioned in the manuscript in order not to confuse readers. Besides, a quick text search at jove.com revealed >600 JoVE articles containing the word “MATLAB”.

7. Please provide the matlab file as a supplemental file.

done

Reviewers' comments:

We thank all reviewers for their time, and their useful comments, suggestions and criticisms. We have carefully addressed all points raised. Please see our responses below.

Reviewer #1:

In the manuscript "Analysis of lipid droplet content in fission and budding yeasts using automated image processing" the author describe how to acquire fluorescence microscopy images of Bodipy-stained cells and how to quantify their number using the software Matlab.This protocol has been tested in three different yeast species: the fission yeasts Schizosaccharomyces pombe and Schizosaccharomyces japonicus, and the budding yeast Saccharomyces cerevisiae. This approach is useful to discover novel genes involved in lipid

droplet homeostasis. The protocol is very clear and easy to follow. I have several minor questions :

Which is the best number of cells that should be analyzed per slide?

The number of cells on a slide is not a critical parameter, as long as the cells are not stacked atop each other. Our workflow does not detect individual cells, but rather discriminates “cell objects” (might be composed of multiple adjacent cells) from the background (stained with fluorescent cell-impermeable dextran). The protocol mentions the fact (3.6) that a cell monolayer should be obtained for imaging, and we now indicate in the Discussion that the number of cells per field of view is not critical.

How many cells have to be analyzed to reach a conclusion?

This will largely depend on the biological system and question under study (e.g., the degree of heterogeneity in the cell population, the expected magnitude of the effect analyzed, etc.). To provide some guidance to the readers, we now state in the figure legends the numbers of cell objects used to derive the representative results.

The authors should comment about Bodipy´s extrusion from the cells. Active PDR pumps extrude Bodipy in living cells (refs 1 and 2)

Thank you for pointing this out. While the references you mentioned deal with export of Nile Red, not BODIPY, it is conceivable that analogous effects may be observed even for BODIPY. Therefore, we now mention this potential issue in the Discussion.

They should introduce matlab. Is this an open software?

We now briefly introduce the MATLAB statistical package in the Introduction, including a note that it is a commercial software.

References

Ivnitski-Steele, I., Holmes, A. R., Lamping, E., Monk, B. C., Cannon, R. D., and Sklar, L. A. (2009). Anal. Biochem. 394, 87-91.

Wolinski, H., and Kohlwein, S. D. (2008). Methods Mol. Biol. 457, 151-163

Reviewer #2:

Manuscript Summary:

This is a technique to analyze the number and volume of lipid droplets in yeast cells using MATLAB.

Major Concerns:

None

Minor Concerns:

Line 163: I prefer 30C to 32C for pombe.

At 32°C fission yeast cells grow faster than at 30°C, reducing the time required to conduct an experiment. We now explain this in the Protocol (2.1.1) and we also mention that some laboratories prefer growing fission yeast at 30°C.

Lines 242-245: It might be useful to give the dimensions of the yeast so that microscopes can be adjusted easily.

done

Line 292: Dirt on the microscope slide can be eliminated by soaking them in CHCl3 prior to adding the yeast.
We have no experience with cleaning empty slides with chloroform, but presumably it works. However, fluorescent debris can be introduced later during the procedure, for example with the yeast suspension. We therefore warn users to check for presence of fluorescent debris. Nevertheless, we now provide description of an optional slide washing procedure in the protocol (3.1).
Lines 300-303: The number of dead cells should be minimal if the reader follows the suggestion of going through at least 2 rounds of division.
We agree, but this is only applicable to some culture conditions, such as exponential growth. For example, if stationary-phase cells are studied, the number of dead cells can be high. That is why we included the text you mention.
Figure 2: Please include error bars.
Barplots in panels B and C in Figures 2-4 show summary metrics for each particular dataset. These are single values, so no error bars are included. We now state explicitly at the beginning of the Representative Results section that each example corresponds to a single biological experiment.
The use of ppc1-88 isn't necessary but they may keep it in if they please. Perhaps a better "control" would be dga1-delta, plh1-delta, are1-delta, and are2-delta, but it's not a big deal.
Thank you for your suggestions. Nevertheless, we decided to keep *ppc1-88* as an example of cells with low LD content to demonstrate the functioning of our workflow with low-signal images. We now explain this in the text.
In this figure, the authors present the volume of LDs but the units confuse me. What are "voxels"? How about nm^3?
Voxels are units of volume (volume pixels, or “3D pixels”) routinely used in analysis of 3D digital images. They can represent different physical sizes depending on the resolution of the 3D image in the x, y and z axes. Therefore, the use of nm3 or similar units would be misleading in this case.
All figures: because this is a high throughput technique, I would like to see cell numbers. Such as we analyzed 1000 cells, etc.
We now state in figure legends the numbers of cell objects used to derive data shown in Figs. 2-4, panels B-E.

Reviewer #3:
Manuscript Summary:
The manuscript by Pincova et al, describes a method for staining and analysing lipid droplets in a range of different yeast species. This technique allows the number, intensity and volume of lipid droplets within yeast to be analysed using a relatively simple staining method. This is an important method, which can be utilised by a range of scientists interested in basic lipid metabolism and industrial applications. The authors show a set of representative data to support their conclusions about the use of this method, however, the data could be enhanced by including some level of statistical analysis.

Major Concerns:

My only major concern is the lack of statistical analysis within the paper. In some cases, especially for the total intensity and volume of the lipid droplets, comments are made about the values e.g. "moderately higher" but it would strengthen the paper if this could be confirmed as statistically significant.
We have now added tests of statistical significance (unpaired Wilcoxon test) for Figures 2-4, panels D-E. The p values are stated in the respective figure legends. All relevant differences between samples described in the main text are highly significant.

Minor Concerns:
Introduction
* Line 52/53 - Reference required for statement about composition of LD
Reference added.
Protocol
* Line 129 - include the final concentration of soybean lectin
done
* Prepare cultivation media
o Explanation for only using chemically defined media (EMM) with S.pombe and not the other yeast species? Published work examining yeast lipids in S. cerevisiae commonly uses chemically defined media (YNB) over YPD.
In line with the scope of the JoVE journal, the manuscript is focused on describing the methods, and only a few sample results are shown. The paper is not meant as a systematic study of yeast LD content. For each yeast species, we chose representative media, growth phases and/or mutants that are already known to affect cellular LD content, to demonstrate the discriminative capabilities of our workflow. We now clarify this at the beginning of the Representative Results section.
We also now describe (1.4.4) the preparation of defined minimal medium for *S. cerevisiae*, so that the readers can decide which medium to use.
* Line 163 - rationale for using 32oC rather than 30oC as the growth temperature for S.pombe?
At 32°C fission yeast cells grow faster than at 30°C, reducing the time required to conduct an experiment. We now explain this in the Protocol (2.1.1) and also mention that some laboratories prefer growing fission yeast at 30°C.
* Line 204 - can further details be given on using a pipette top to spread the coating solution?
done
* Line 168 - could the number of hours be indicated rather than just commenting on late afternoon.
done
* Line 221 - include comment on resuspending the cells in remaining supernatant.
done
Representative results
* Line 337 - Needs to be clear that Fig 1 summarises the workflow for S.pombe not S. cerevisiae.
The text was changed accordingly.
* Line 342 - what phase of growth was used for the experiments shown in Fig 2. This should be indicated in the text and the figure legend.

done

* Line 352 - the S.pombe ppc1-88 cells where grown at 36oC. Where the appropriate controls undertaken for this i.e. growing the wildtype cells are 36oC. If so, was there any changes in lipid droplets at this higher temperature?

Thank you for pointing this out. We did not analyze WT cells at 36°C for this manuscript. The *ppc1-88* mutant grown at restrictive temperature was meant solely as an example of cells with low BODIPY signal to demonstrate the functionality of our workflow on low-signal images. We now explain this in the text.

Figures and Table legends

* Figure 1 - needs to be clear that this is for fission yeast i.e. S.pombe/S.japonicus not S. cerevisiae.

We changed the figure legend accordingly.

* Figure 2 - indicate the phase of growth and growth temperatures.

done

Manuscript with tracked changes

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JoVE_tracked.doc

---
xxx_cells.csv
---
fileName - name of saved CSV file
id - cell object identifier
Area3D - sum of areas in all slices in which the cell object was identified [voxels]
Area2D - maximum projection area of the cell object in xy plane [voxels]
IntensitySumBlue - integrated intensity of the cell object in blue channel
IntensityMeanBlue - mean intensity of the cell object in blue channel
IntensityMedianBlue - median intensity of the cell object in blue channel
CentroidX - x coordinate of the cell object centroid
CentroidY - y coordinate of the cell object centroid
CentroidZ - z coordinate of the cell object centroid
numDots - number of identified LDs in the cell object
dotsId - identifiers of LDs located in the cell object
Dot_intensity_sum_per_cell - integrated intensity of all identified LDs in the cell object in green channel

---
xxx_dots.csv
---
fileName - name of saved CSV file
id - LD identifier
Area3D - sum of areas in all slices in which the LD was identified [voxels]
IntensitySum - integrated intensity of the LD in green channel
IntensityMean - mean intensity of the LD in green channel
IntensityMedian - median intensity of the LD in green channel
CentroidX - x coordinate of the LD centroid
CentroidY - y coordinate of the LD centroid
CentroidZ - z coordinate of the LD centroid
idCellObject - cell object identifier in which the LD is located

MATLAB main script

MATLAB functions 2

MATLAB functionst 3

MATLAB functions 4

MATLAB functions 6