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Journal

Current Protocols in Microbiology, 53(1)

ISSN

1934-8525

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Publication Date

2019-06-01

DOI

10.1002/cpmc.76

Peer reviewed

HHS Public Access

Author manuscript

Curr Protoc Microbiol. Author manuscript; available in PMC 2020 March 05.

Published in final edited form as:

Curr Protoc Microbiol. 2019 June ; 53(1): e76. doi:10.1002/cpmc.76.

Monitoring Phenotypic Switching in *Candida albicans* and the Use of Next-Gen Fluorescence Reporters

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Abstract

Candida albicans is an opportunistic human fungal pathogen that is able to cause both mucosal and systemic infections. It is also a frequent human commensal, where it is typically found inhabiting multiple niches including the gastrointestinal tract. One of the most remarkable features of *C. albicans* biology is its ability to undergo heritable and reversible switching between different phenotypic states, a phenomenon known as phenotypic switching. This is best exemplified by the white-opaque switch, in which cells undergo epigenetic transitions between two alternative cellular states. Here, we describe assays to quantify the frequency of switching between states, as well as methods to help identify cells in different phenotypic states. We also describe the use of environmental cues that can induce switching into either the white or opaque state. Finally, we introduce the use of mNeonGreen and mScarlet fluorescent proteins that have been optimized for use in *C. albicans* and which outperform commonly used fluorescent proteins for both fluorescence microscopy and flow cytometry.

Keywords

Candida albicans; cell identity; fluorescent reporters; gene expression; heritable states; phenotypic switching

INTRODUCTION

Many microbial species have evolved the ability to generate phenotypic diversity. Phenotypic heterogeneity can have a number of beneficial roles including ‘bet hedging,’ whereby a subset of isogenic cells within a population are pre-adapted to certain environments, thereby enabling rapid adaptation to fluctuating conditions (Martins & Locke, 2015). Phenotypic variation has been extensively evaluated in microbial pathogens, where processes such as phase variation and phenotypic switching can promote immune evasion or direct tissue tropisms (Noble, Gianetti, & Witchley, 2017; van der Woude & Baumler, 2004;

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Veening, Smits, & Kuipers, 2008). An understanding of these mechanisms is therefore critical to defining how microbial pathogens interact with the host.

Candida albicans is an opportunistic human fungal pathogen that is a normal component of the microbiota, often found inhabiting the mucosal membranes as well as the gastrointestinal (GI) and urogenital tracts (Ghannoum et al., 2010; Odds, 1988). However, it is capable of causing a variety of opportunistic infections including both mucosal infections as well as life-threatening systemic disease (Edmond et al., 1999; Pfaller et al., 2012). These infections can arise due to antibiotic use, organ transplantation, steroid treatment, or immunodeficiency (Colombo et al., 2003; Fukushima et al., 2001; Imam et al., 1990). Even with antifungal treatment, mortality rates with disseminated candidiasis are high at ~37% to 44% (Leroy et al., 2009; Moran, Grussemeyer, Spalding, Benjamin, & Reed, 2010).

A key aspect of the flexible lifestyle of *C. albicans* is the ability to adapt to environmental cues by both genetic and epigenetic mechanisms. In particular, cells can stochastically and reversibly transition between two forms, white and opaque, that are distinguished by their colony or cellular morphologies (Slutsky et al., 1987) as shown in Figure 1. The mechanism of white-opaque switching has been intensively studied and involves a transcriptional circuit centered on two factors, Efg1 and Wor1, which operate as a binary toggle switch (Hernday et al., 2013; Huang et al., 2006; Sonneborn, Tebarth, & Ernst, 1999; Srikantha et al., 2006; Srikantha, Tsai, Daniels, & Soll, 2000; Zordan, Galgoczy, & Johnson, 2006; Zordan, Miller, Galgoczy, Tuch, & Johnson, 2007). Efg1 expression promotes formation of the white state whereas Wor1 is antagonistic to Efg1 and promotes formation of the opaque state (Fig. 1). A number of other transcription factors impinge on Efg1 and Wor1 to modulate switching frequencies (Alkafeef, Yu, Huang, & Liu, 2018; Lohse & Johnson, 2016; Lohse et al., 2016; Lohse et al., 2013; Vinces & Kumamoto, 2007; Zordan et al., 2007). In addition to this transcription factor network, chromatin-level control is also important with a number of histone-modifying enzymes shown to regulate the white-opaque switch (Hnisz, Schwarzmuller, & Kuchler, 2009; Stevenson & Liu, 2011; Xie, Jenull, Tscherner, & Kuchler, 2016).

In this article, we provide several methodologies to analyze white/opaque states and cell state switching in *C. albicans*. This includes protocols for the quantification of white-opaque switching, the use of fluorescent reporters to aid in identification of the two cell states, and related methods for the manipulation of cells between white and opaque states. In addition, we describe *Candida*-optimized next-generation fluorescent proteins mNeonGreen and mScarlet, which significantly outperform green fluorescent protein (GFP) and mCherry, respectively. This collection of protocols will allow the researcher to make a detailed analysis of phenotypic states in *C. albicans*.

BASIC PROTOCOL 1

PHENOTYPIC SWITCHING IN A COLONY-SECTORING ASSAY ON AGAR PLATES

The standard white-opaque phenotypic switching assay in *C. albicans* involves analysis of individual colonies to determine their cellular state with switching events manifesting as a change in colony morphology.

A pure population of cells in one state are diluted and spread to produce ~50 to 100 single colonies on agar plates (Fig. 2A). A large sector indicates that a cell switched identity during the first few divisions after plating and this state was stably inherited by its progeny resulting in a “pie slice” of the colony consisting of cells of a different state. As opaque colonies tend to grow wider and flatter, an opaque sector in a white colony will often cause a deformation of the colony edge, which can be a useful visual cue while scoring switching events (Fig. 2B). This experiment can be performed in either direction to determine white-to-opaque or opaque-to-white switching frequencies.

Materials

Synthetic complete dextrose (SCD) liquid medium (see recipe)

Glycerol stocks of *C. albicans* strain(s), frozen in either white or opaque state

SCD agar plates (standard size of 100 mm × 15 mm; see recipe)

Sterile flat toothpicks

Cuvettes and spectrophotometer, or hemocytometer

Light microscope (with a 40× objective)

Stereomicroscope

4-mm glass beads (VWR, cat. no. 26396–563 or Thermo Fisher Scientific, cat. no. S800241)

Tally counter

1. Streak *C. albicans* cells to single colonies onto SCD plates from glycerol stocks using a sterile toothpick. Culture 3 days at room temperature or until the state of each colony can be readily determined by colony morphology.

   Cultures frozen in the opaque state will predominantly form colonies of opaque cells on solid medium at 22°C. However, opaque cells will switch back to predominantly white cells if incubated at 30°C or entirely white cells at 37°C (see Basic Protocol 2). This can be useful for generating a pure white population of a strain such as WO-1 that shows high rates of white-to-opaque switching.

2. Using a stereomicroscope, evaluate individual colonies and determine whether they are white (shiny, dome-shaped) or opaque (flat, dull).

   Examine cells directly from colonies by microscopy (40× objective or higher) if unsure whether colonies consist of white (round) or opaque (elongated) cells.

3. Pick independent colonies with sterile toothpicks and resuspend (separately) in 1 ml liquid SCD medium.

4. Measure OD_{600} of cell suspension using cuvettes in a spectrophotometer.

Dilute cell suspension in water to get the OD into the linear range of a spectrophotometer. The cells from one colony resuspended in 1 ml of water will typically require between a 1/20 and a 1/50 dilution to accomplish this.

5. Calculate cell density of cell suspension; OD_{600} = 1 is equivalent to ~2 × 10^7 cells/ml.

   Be aware that different strain backgrounds or different spectrophotometers could give different cell densities per OD_{600} value. Also note that an adjustment for opaque cells (plating 1.5 or 2 times the volume) is necessary due to their larger size than white cells. Cell density can also be checked using a hemocytometer to establish cells/ml to provide a more precise measure of culture density.

6. Make an appropriate cell dilution in water or SCD so that ~50 to 100 cells can be plated in a volume of 100 μl or less.

   For example, making a cell suspension of 2 × 10^7 cells/ml followed by two 1/100 dilutions to 2 × 10^3 cells/ml will allow plating of ~100 white cells in 50 μl and 100 opaque cells in 75 to 100 μl.

7. Plate multiple SCD plates for each individual colony analyzed to obtain a sufficient number of switching events.

   Spreading cells with sterile 4-mm glass beads is preferable to using a glass or plastic cell spreader as beads give a more uniform distribution of colonies and do not mark the surface of the plate. These beads are cleaned by soaking in dilute bleach for 30 min, thoroughly rinsed, allowed to air dry, and autoclaved in 100-ml glass medium bottles for reuse.

8. Incubate plates 7 days at room temperature (~22°C). Using a tally counter, determine the number of colonies on each plate. Using a stereomicroscope, examine each colony for sectors indicating a switching event. Evaluate for whole colonies that have switched phenotype as well as for sectors indicating switching to the alternative cell state.

   We have observed that some C. albicans strains appear to be very sensitive to temperature so that even small fluctuations in room temperature can affect switching frequencies. For this reason, it is ideal if experiments can be performed in refrigerated incubators that can be set to 22°C (or 25°C) to ensure greater reproducibility. However, we have also performed switching assays on the benchtop when necessary.

9. Calculate switching frequency as the number of whole colonies and colonies with sectors that have switched phenotype, divided by the total number of colonies.

For manipulations that increase the rate of switching, it can be useful to record switching frequencies separately for fully switched versus sectored colonies. Thus, both the “fully switched” and “fully switched + sectored” numbers can be reported.

The appearance of new sectors will continually emerge over time so it is important to analyze all plates after a defined period of incubation. If unsure if a sector represents a change in phenotype, cells from the sector can be analyzed by microscopy or by subculturing to a separate agar plate.

ALTERNATE PROTOCOL 1

ANALYSIS OF SWITCHING ON MEDIUM CONTAINING PHLOXINE B

While we and others perform white-opaque switching assays on SCD medium, the dye phloxine B has often been included in culture media as it preferentially stains *C. albicans* colonies in the opaque state (Fig. 3A). Opaque colonies are therefore pink in the presence of phloxine B whereas white colonies remain white or are only slightly pink (Anderson & Soll, 1987). It is worth noting that phloxine B is somewhat toxic to some yeast species so is perhaps best used for terminal experiments (not for isolation of opaque cells for other experiments; Mutoh, Kawabata, Nakagawa, & Kitajima, 2005).

Materials

SCD liquid medium (see recipe)

SCD plates supplemented with 5 μg/ml phloxine B (Sigma-Aldrich, cat. no. P2759; from a 10 mg/ml stock in DMSO)

Glycerol stocks of *Candida albicans* strain(s), frozen in either white or opaque state SCD agar plates (see recipe standard size of 100 mm × 15 mm)

Sterile flat toothpicks

Cuvettes and spectrophotometer, or hemocytometer

Light microscope with a 40× (or higher) objective

Stereomicroscope

4-mm glass beads (VWR, cat. no. 26396–563 or Thermo Fisher Scientific, cat. no. S800241)

Tally counter

1. Perform steps 1 through 6 of Basic Protocol 1.
2. Plate cells for single colonies on medium supplemented with 5 μg/ml phloxine B.

   Phloxine B has been used to monitor switching on YPD plates (Tao et al., 2014) as well as on Lee’s amino acid rich medium (Lee, Buckley, & Campbell, 1975) supplemented with zinc and arginine

(Anderson & Soll, 1987; Bedell & Soll, 1979). Phloxine B can also distinguish white and opaque colonies in the related Candida species Candida dubliniensis (Pujol et al., 2004) and Candida tropicalis (Xie et al., 2012), where in each case opaque colonies selectively stain with this dye.

3. Incubate plates 7 days at ~22°C and evaluate for whole colonies that have switched phenotype and for sectors, as described above (step 2 and Basic Protocol 1, step 9).

ALTERNATE PROTOCOL 2

ANALYSIS OF WHITE-OPAQUE SWITCHING ON CHROMagar CANDIDA MEDIUM

Phloxine B has been extensively used for monitoring white-opaque switching, yet we have found that CHROMagar Candida medium is also an excellent chromogenic medium for distinguishing *C. albicans* cells in different phenotypic states. As shown in Figure 3B, white and opaque cells generate colonies that are light green and blue/purple, respectively. This commercial medium is therefore an alternative to distinguish white/opaque cell states. In our experience, clinical backgrounds besides SC5314 vary in the shade of green taken on by white colonies but can still be readily distinguished from opaque. CHROMagar is not able to readily distinguish *C. tropicalis* white and opaque colonies. The white-to-opaque switching frequency on CHROMagar is somewhat lower than for assays performed on SCD medium.

Materials

SCD liquid medium (see recipe)

Glycerol stocks of *C. albicans* strain(s), frozen in either white or opaque state

SCD agar plates (see recipe standard size of 100 mm × 15 mm)

CHROMagar Candida plates (Drg International)

Sterile flat toothpicks

Cuvettes and spectrophotometer, or hemocytometer

Light microscope with a 40× objective

Stereomicroscope

4-mm glass beads (VWR, cat. no. 26396–563 or Thermo Fisher Scientific, cat. no. S800241)

Tally counter

1. Perform steps 1 through 6 of Basic Protocol 1.
2. Instead of plating cells for single colonies on SCD plates, plate 50 to 100 cells onto CHROMagar Candida plates.

3. Allow colonies to grow for 7 days at room temperature (~22°C) and evaluate both for whole colonies that have switched phenotype and for sectors indicating switching to the alternative cell state.

BASIC PROTOCOL 2

ENVIRONMENTAL CONDITIONS TO PROMOTE WHITE-TO-OPAQUE SWITCH

While stochastic white-to-opaque switching occurs on standard laboratory medium, it is often useful to induce switching in order to more efficiently obtain colonies in the opaque state. Several conditions can be used to induce this switch with the most common one in our laboratory being growth on medium containing N-acetyl-D-glucosamine (GlcNAc) at room temperature, as previously reported (Huang et al., 2010).

Additional Materials (also see Basic Protocol 1)

C. albicans strain(s), American Type Culture Collection (sc5314 MTLa/alpha, ATCC-MYA2876)

Lee's plus N-acetyl-D-glucosamine (GlcNAc) medium (see recipe)

Stereomicroscope

CO_2 incubator (optional)

1. Streak *C. albicans* cells onto SCD plates at room temperature (~22°C) from glycerol stocks. Culture 3 to 5 days.
2. Re-streak strains for single colonies on Lee's medium supplemented with GlcNAc.

   Streak densely as the frequency of switching is still relatively low in our hands. Alternatively, cells can be grown on synthetic complete (SC) plates supplemented with GlcNAc (SCG plates) instead of dextrose, which are simpler to make than Lee's supplemented medium.
3. Culture 3 to 5 days at room temperature.
4. Examine using a stereomicroscope looking for sectors or whole colonies that have switched from the white state to the opaque state (Fig. 4).

   Opaque colonies are often detectable by their wrinkled, fuzzy appearance on this medium.
5. Strike out cells from potential opaque sectors on SCD medium to obtain colonies containing a pure population of opaque cells. Streak cells from potential opaque sectors again to single colonies on SCD medium to obtain colonies containing a pure population of opaque cells.

   Other conditions that induce white-to-opaque switching include incubation on supplemented Lee's medium in a CO_2 incubator at 25°C for 5 days (5% to 20% CO_2, with higher concentrations

inducing higher levels of switching; Huang, Srikantha, Sahni, Yi, & Soll, 2009). CO_2 can also further enhance white-to-opaque switching in cells grown with GlcNAc, as these stimuli act via independent signaling pathways (Huang et al., 2010).

BASIC PROTOCOL 3

PCR AMPLIFICATION OF CONSTRUCTS FOR FLUORESCENT REPORTERS

Cell and colony morphologies are often reliable phenotypes to distinguish white and opaque states in *C. albicans*. However, it is frequently beneficial to analyze cell state-specific gene expression which can be achieved using fluorescent protein (FP) reporters. For example, expression of the *WH11* and *OP4* genes have been repeatedly used to define white and opaque states, respectively (Morrow, Srikantha, Anderson, & Soll, 1993; Srikantha & Soll, 1993). These reporters have been used both in the standard laboratory SC5314 strain as well as in other clinical isolates of *C. albicans*.

The constructs used for *WH11* and *OP4* reporters can be directly PCR amplified from plasmids containing a fluorescent reporter gene together with a selectable marker gene. For *WH11* and *OP4* reporters, the FP is integrated to replace one copy of the target gene by homologous recombination, thus putting it under the transcriptional control of the endogenous promoter. A number of choices are available for both the FP and the selectable marker. Codon optimized GFP and mCherry have been developed for *C. albicans*; this is necessary as *C. albicans* uses a non-standard genetic code (Santos & Tuite, 1995). Our standard vectors are derivatives of the *pSFS2A* "SAT1-flipper" plasmid which allows the nourseothricin resistance marker to be recycled for additional rounds of transformation (Reuss, Vik, Kolter, & Morschhauser, 2004). Here, a maltose inducible promoter controls expression of an *FLP* recombinase, which induces recombination between two FRT sites flanking the nourseothricin resistance marker as well as the *FLP* recombinase gene itself. Therefore, by simply incubating the resulting strains on medium containing maltose as a carbon source the resistance marker is excised.

These vectors can also be used to fluorescently tag the transcription factors (TFs) regulating the white-opaque switch. This is typically done by designing an FP construct to integrate at the 3′ end of the TF open reading frame and excluding the stop codon (Fig. 4). Using the template homology indicated in the oligonucleotides in Table 1 will ensure that the correct reading frame is maintained.

Materials

TaKaRa Ex Taq DNA polymerase

dNTP mix

Long oligonucleotides for generating transformation cassette

Optional: DNA cleanup kit (DNA Clean & Concentrator, Zymo Research, or equivalent)

Optional: 100% ethanol

Optional: 3 M sodium acetate, pH 5.2

Thermocycler

Agarose electrophoresis equipment

Agarose gel casting apparatus

Electrophoresis power supply

1. Design oligonucleotides that anneal to the target plasmid containing the FP and selectable marker (see Table 1 and Hernday, Noble, Mitrovich, & Johnson, 2010): Oligonucleotides should contain at least 70 nucleotides (nts) of homology to the target gene (oligos are therefore ~100 nts long). For the forward oligo, the homology should end at the base immediately before the start codon of the gene (Figure 5A). Take homology for the reverse oligo from the intergenic region 3′ to the open reading frame (ORF). Find further information on oligo design in Hernday et al., 2010.

   We routinely use 100 nt oligos without PAGE purification for the PCR amplification of tagging cassettes and find that the majority of clones where integration has been confirmed show the expected fluorescence signal.

   Several of the vectors we use for PCR templates for GFP (pADH76; Lohse & Johnson, 2016) and mCherry (pADH77) are designed such that the same pair of oligonucleotides can be used to amplify different fluorophore tags when targeting the same C. albicans gene. Similarly, the same oligos can be used with plasmid pADH34 for tagging genes with a 13×myc epitope tag or with plasmid pADH52 for tagging genes with a 6×his-FLAG tag (Hernday et al., 2010).

2. Perform 35 cycles of PCR amplification using the designed primers with Ex Taq polymerase.

   Note that to minimize the chances that DNA mutations will be present, independent PCR reactions are often performed and pooled for transformations.

   93°C for 3 min;

   98°C for 10 sec;

   55°C for 30 sec;

   72°C for 4 min;

   55°C for 30 sec, 34 times;

   72°C for 10 min;

   Hold at 10°C.

Ex Taq (Takara) works well for these reactions although Phusion (Thermo Fisher Scientific) or other high fidelity polymerases can also be used.

3. Analyze 3 µl PCR product on a 0.8% agarose gel for a product that is 5 kb in length; electrophoresis at 120 V for 25 min is sufficient to resolve DNA of the correct size relative to molecular weight markers.

   Note that spectrophotometric measurements to quantify a PCR product will not be accurate as unincorporated dNTPs and oligos in the solution will also absorb. Run the PCR product on an agarose gel and compare against a molecular weight marker where the bands are of known concentration (this information is available on the manufacturer's product insert of the molecular weight marker). Generating 5 to 10 µg of PCR product often requires pooling multiple reactions, however, the DNA must be concentrated into a smaller volume (30 to 50 µl) for the subsequent transformation. This is accomplished either through the use of a PCR cleanup kit (DNA Clean & Concentrator or equivalent) or by ethanol precipitation.

4. Optional: Ethanol precipitate DNA. Add one-tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of 100% ethanol. Mix well and place at −80°C for >20 min. Centrifuge at 16,100 × *g* for 10 min at 4°C in a microcentrifuge and remove supernatant.

   The precipitated DNA should be visible as a small white pellet.

   Remove as much liquid as possible but do not disturb the pellet. Wash pellet with 0.5 ml of ice-cold 70% ethanol, centrifuge 10 min at 4°C, and remove supernatant. Air dry DNA pellet by leaving the tube open on the bench 20 to 30 min until residual liquid is evaporated then resuspend in 50 µl H_2O.

   Do not dry the DNA pellet in a heatblock as this can cause the DNA to be extremely difficult to resuspend.

BASIC PROTOCOL 4

TRANSFORMATION OF *C. ALBICANS* STRAINS WITH FLUORESCENT REPORTERS TO DISTINGUISH WHITE-OPAQUE STATES

Here, we describe a lithium acetate/PEG/heat-shock transformation protocol for integration of DNA cassettes to generate fluorescent reporters and fluorescent protein fusions.

Materials

C. albicans strain to be transformed

YPD medium (see recipe)

Solutions for transformations, e.g., lithium acetate (LiOAc)/TE buffer (see recipe); PEG/LiOAc/TE buffer (see recipe)

Sonicated salmon sperm DNA, 10 mg/ml (Sigma-Aldrich, cat. no. D1626)

YPD medium plus nourseothricin (see recipe; for plates)

Culture tubes

Centrifuge

Microcentrifuge tubes

Fluorescence microscope (e.g., Zeiss Axiovert inverted microscope with 63× objective)

Microscope slides

Cover slips

1. Streak the *C. albicans* strain to be transformed onto SCD or YPD plates from a frozen stock and incubate at room temperature (~22°C) or at 30°C. Culture up to 7 days.

   Note that the transformation protocol described here induces opaque cells to switch to the white state, so it is not possible to transform opaque cells and to immediately analyze them in this phenotypic state.

2. Inoculate *C. albicans* cells into 3 ml YPD medium in a culture tube. Grow at 30°C overnight with shaking or on a roller drum.

3. Take 100 µl from the overnight culture and inoculate 10 ml YPD in a culture tube. This volume of culture will provide enough cells for two transformations. Grow at 30°C, 5 hr.

   Transformation efficiency is affected by the growth state of the cells used. Diluting a saturated overnight culture into fresh medium and allowing the cells to divide several times will ensure that the population is in mid-log phase for more efficient transformation.

4. Spin down the 10-ml cultures in a bench top centrifuge for 5 min at 1300 × *g*.

5. Decant supernatant and resuspend cell pellet in 5 ml LiOAc/TE solution. Centrifuge 5 min at 1300 × *g*.

6. Decant supernatant, resuspend cell pellet in 1 ml LiOAc/TE solution, and transfer to a 1.5-ml microcentrifuge tube.

7. Centrifuge at maximum speed in a microcentrifuge 30 sec.

8. Pipet off supernatant and resuspend cell pellet in 200 µl LiOAc/TE solution.

9. Transfer 100 µl of cells in LiOAc/TE to a fresh 1.5-ml microcentrifuge tube and add 5 µl of 10 mg/ml salmon sperm DNA (ssDNA; pre-boil ssDNA 2

min and snap cool on ice) and 5 to 10 μg PCR-amplified DNA construct in no more than 50 μl of liquid (see Basic Protocol 3).

Note that snap-cooled aliquots of ssDNA can be stored at −20°C and thawed immediately before use without re-boiling and snap cooling.

10. Add 1 ml LiOAc/TE/PEG solution, invert several times to mix, and leave at room temperature overnight.

11. Heat-shock cells at 42°C, 1 hr, centrifuge at maximum speed 1 min in a microcentrifuge, and remove supernatant.

We treat the cells gently at this point. Resuspend the pellet by gently pipetting up and down to minimize sheer. Do not vortex.

12. PCR constructs for tagging *WH11* and *OP4* are described in Basic Protocol 3. These constructs utilize the *SAT1* gene, which provides resistance to the selectable marker nourseothricin. Resuspend cell pellets in 1 ml YPD, transfer to a disposable culture tube, and incubate 4 hr at 30°C with shaking or rolling.

This recovery step allows time for the cells to express the SAT1-resistance marker before they are placed under selection on nourseothricin-containing medium. This recovery step is not necessary for genetic markers encoding for amino acid biosynthetic genes.

13. Transfer the 1-ml cultures to 1.5-ml microcentrifuge tubes, centrifuge cells at maximum speed 1 min in a microcentrifuge, pipet off 900 μl supernatant, and resuspend cells in remaining 100 μl.

14. Plate cells onto YPD containing 200 μg/ml nourseothricin.

15. Incubate 2 to 3 days at 30°C; nourseothricin-resistant colonies will grow large enough to pick in this time. Patch eight to sixteen colonies from each transformation onto a fresh YPD plus nourseothricin plate.

Transformants are kept under selection to prevent the growth of nourseothricin-sensitive cells whose growth has been inhibited by the drug as they have not acquired the resistance marker but are still viable.

16. Analyze colonies by PCR (see Basic Protocol 3).

17. Confirm that reporter or fluorescent fusion is intact by fluorescence microscopy.

WH11 reporters should only be expressed in the white state whereas OP4 reporters and Wor1 fusions are specific for the opaque state. EFG1 fusions are more highly expressed in the white state than the opaque state.

We use a Zeiss Axiovert inverted microscope with a 63× objective for differential interference contrast (DIC) microscopy. GFP is excited using a Colibri 470 to 475 nm LED and Zeiss filter set #38. mCherry is excited using a HXP120C halogen lamp and Zeiss filter set #45 (Texas Red).

Other promoters can be used as fluorescence reporters to distinguish white/opaque states such as SAP1 which is specifically expressed in opaque cells (Miller & Johnson, 2002; White, Miyasaki, & Agabian, 1993).

Optional: The nourseothricin resistance marker can be excised allowing further rounds of transformation using the same genetic marker. This is accomplished by streaking the cells on SC or YP medium containing 2% maltose as the sole carbon source (no glucose) to activate the MAL2 promoter that controls expression of the FLP recombinase gene. This gene product induces recombination between two FRT sites, removing the FLP gene and the marker from the genome. Following 5 to 7 days growth on maltose plates at room temperature, cells are struck to single colonies on YPD. Single colonies are then patched on plates with and without nourseothricin to identify those which have lost the resistance marker.

BASIC PROTOCOL 5

COLONY PCR FOR CONFIRMATION OF CASSETTE INTEGRATION

We routinely screen colonies by PCR to confirm that integration of DNA constructs has occurred in the correct locus. Our oligo sequences are chosen so that one member of the pair anneals to the DNA construct used in the transformation (e.g., for pSFS2A Sat1-Flipper-based plasmids we use the oligos listed in Table 2) and the other typically anneals to genomic DNA a short distance away (300 to 500 bp) from the end of the homology present in the targeting construct. Thus, a PCR product will only be generated if the cassette has integrated at the appropriate genomic location.

Materials

Taq polymerase

10× colony PCR buffer (see recipe)

25 mM magnesium chloride ($MgCl_2$)

dNTP mix

Oligonucleotides for checking junctions of integration (Table 2)

PCR thermocycler

Agarose electrophoresis tank

Agarose gel casting apparatus

Electrophoresis power supply

1. Set up reactions as follows in 0.2-ml PCR strip tubes:

10× colony PCR buffer	2.5 µl
25 mM $MgCl_2$	1.5 µl
10 mM dNTP	2 µl
Forward oligo (100 µM)	0.1 µl
Reverse oligo (100 µM)	0.1 µl
Water	18.3 µl.

Typically, initial screens will examine at least eight independent colonies per transformation. A master mix of the above solutions is made up and should include enough for one or more extra reactions to have sufficient mix for distribution between PCR tubes. Include an additional reaction for PCR from the parental strain for a negative control.

2. Transfer a small amount of cells to each tube using a sterile pipet tip such that the PCR mix is only slightly turbid (too many cells can inhibit the reaction). Crush cells on the pipet tip against the side of the tube with some force to ensure a portion of the cells are lysed and their genomic DNA released.

You should be able to hear and feel the tip and Eppendorf tube squeaking as they rub together.

Alternately, a small amount of Candida can be transferred to a microcentrifuge tube containing 100 µl of 20 µM NaOH and incubated at 99°C for 10 min. Of this sample, 5 µl can then be added to each PCR reaction prior to thermocycling (Nguyen, Quail, & Hernday, 2017). Be sure to adjust the volume of water in the recipe listed above so that the final volume equals 25 µl.

3. Add *Taq* polymerase (0.5 µl) to each tube after breaking the cells. Place tubes in a thermocycler.

95°C for 3 min;

95°C for 30 sec;

55°C for 30 sec;

68°C for *x* min (i.e., 1 min per kb of target DNA; see note below);

55°C for 30 sec, 34 times;

68°C for 10 min;

Hold at 10°C.

For PCR amplification checking the left flank, “pSFS2A left reverse” is positioned ~1100 bp from the 5′ end of the cassette, reading through the fluorescent protein tag (see Figure 5A). Therefore, if the forward check oligo is 500 bp upstream from the integration site, the PCR product will be 1600 bp, requiring a 1 min 45 sec extension time per cycle. “pSFS2A right forward” is ~400 bp from the 3′ end of the cassette.

BASIC PROTOCOL 6

GENERATION OF *MTL* HOMOZYGOUS DERIVATIVES OF *C. ALBICANS MTL*a/α STRAINS

The white-to-opaque switch is inhibited by a complex formed by the ***a1*** and ***α2*** gene products, which are encoded at the mating type-like (*MTL*) locus in diploid *C. albicans* cells. As a result, *MTL***a**/α strains typically cannot form the opaque state whereas both *MTL***a**/**a** and *MTL*α/α strains are competent for white-to-opaque switching (Lockhart et al., 2002; Miller & Johnson, 2002). Given that the majority of natural *C. albicans* isolates are *MTL***a**/α (Legrand et al., 2004; Lockhart et al., 2002), it is often necessary to convert an *MTL***a**/α strain to an *MTL* homozygous strain to be able to examine the opaque form of a strain. This is typically done by one of two methods: (1) genetic deletion of one of the two *MTL* idiomorphs by homologous recombination using a selectable marker (Figure 6A), or (2) growth on sorbose medium. Cells plated on sorbose as a carbon source are unable to grow unless they become monosomic for chromosome 5 (Chr5), which contains several repressors of sorbose metabolism as well as the *MTL* locus. Re-plating these aneuploid cells on glucose-containing medium allows them to re-duplicate Chr5 resulting in a euploid cell that is homozygous for Chr5 (Janbon, Sherman, & Rustchenko, 1998; Magee & Magee, 2000; Figure 6B). We describe both of these approaches below.

Additional Materials (also see Basic Protocol 3)

*MTL***a**/α *C. albicans* strains (American Type Culture Collection; sc5314 *MTL*a/alpha, ATCC-MYA2876)

Plasmids for targeting the *MTL* locus for deletion (pRB101 or pRB102; Alby & Bennett, 2009)

*Apa*I and *Sac*I restriction enzymes

Reagents for transformations (see Basic Protocol 3)

SCD or YPD plates

YPD plus nourseothricin plates (see recipe)

Sorbose plates

Delete MTL idiomorph

1. Streak *C. albicans* cells onto SCD or YPD plates at 30°C from glycerol stocks. Culture 2 to 3 days.
2. Digest plasmid pRB101 (targets *MTL***a**) or pRB102 (targets *MTL*α) with both *Apa*I and *Sac*I enzymes.

ApaI cuts optimally at 25°C, and SacI at 37°C, so it is convenient to do these digestions sequentially in a thermocycler overnight. Add both enzymes and then incubate for 4 hr at 25°C followed by 4 hr at 37°C, then hold the reactions at 10°C.

3. Analyze 1 μl digested DNA by electrophoresis to ensure that the enzymes have cut efficiently producing two bands: A 5200 bp band which contains the *SAT1* flipper cassette flanked by *MTL* homology and a 3300 bp band which represents the remainder of the plasmid.

   It is not necessary to gel purify the larger band.

4. Ethanol precipitate DNA (see Basic Protocol 3, step 4).

5. Transform digested plasmid into the target strain using the transformation protocol described in Basic Protocol 3.

6. Test for deletion of either *MTL***a** or *MTL*α by colony PCR against *MTL*-specific alleles of *PAP* or *OBP* genes (see Table 3).

   Note that although the homology in pRB101 and pRB102 favors targeting of one MTL locus over the other, the “incorrect” MTL locus is often deleted so that it is worthwhile testing whether either MTL locus has been deleted (see Table 4 for oligonucleotide sequences used for colony testing).

   An analogous approach is to use the vector pJD1 (an ARG4-marked cassette for making MTLα deletions), which is also effective for targeting the MTL in Arg negative strains (Lin et al., 2013).

Generate MTL homozygous strains by growth on sorbose medium

7. Streak *MTL***a**/α *C. albicans* cells onto SCD or YPD plates at 30°C from glycerol stocks. Culture 2 to 3 days.

8. Re-streak cells onto sorbose medium and incubate at 30°C, 1 week.

   These should be struck out densely as most cells do not grow on sorbose medium. Also note that auxotrophic strains will require the necessary amino acid be added to the sorbose medium.

9. Pick colonies from sorbose and re-streak to quadrants on YPD at 30°C.

   We find that cells that have lost chromosome 5 tend to produce small colonies initially (small colonies observed for first 3 days) but then larger growing colonies arise within these subpopulations which are then selected for further analysis. We assume that the faster-growing colonies arise due to duplication of the monosomic chromosome 5 back to a disomic form, as previously described (Janbon et al., 1998). Many colonies recovered from sorbose have not undergone loss of an MTL locus so this method of analyzing growth on YPD helps narrow the search for the desired colonies.

10. Perform colony PCR on select colonies to determine if the *MTL***a** or *MTL*α locus has been lost. This is achieved using PCR with oligonucleotides specific to *MTL* alleles of the *OBP* or *PAP* genes (Table 4).

    In general, we use the method of genetically deleting one of the MTL alleles to generate strains that are white-opaque switching competent. The sorbose selection method often works, yet it is possible that other genetic changes occur using this selection in addition to (or alternative to) the loss of one copy of chromosome 5 (Janbon, Sherman, & Rustchenko, 1999).

SUPPORT PROTOCOL

GENERATION OF STABLE FROZEN STOCKS OF OPAQUE CELLS

The standard method used for freezing *C. albicans* strains at −80°C involves taking cells directly from agar plates, resuspension in a freezing medium, and placing vials directly at −80°C. However, opaque cells stored using this method are typically in the white state when recovered from the freezer. We therefore use an alternative method for storing opaque cells, which ensures that many of these cells retain the opaque state when stored at −80°C.

Materials

Opaque *C. albicans* strain (see Basic Protocol 2)

SCD medium

Sterile 50% glycerol

Culture tubes

2-ml cryogenic storage vials

Roller drum at room temperature

−80°C freezer

1. Take cells from a pure colony of opaque cells grown on an agar plate and inoculate 3 ml SCD medium in a culture tube.
2. Culture overnight at room temperature with shaking or on a roller.
3. Take 1 ml saturated cell culture and mix with 1 ml of 50% sterile glycerol in a cryogenic vial.
4. Mix well and place in a storage box at −80°C.

   Cells can be stored indefinitely at −80°C. In cases where a large number of strains are being frozen, this protocol can also be used in a 96-well plate format with reduced volumes.

REAGENTS AND SOLUTIONS

For common stock solutions, see Current Protocols, 2007.

Colony PCR buffer, 10×

100 mM Tris, pH 9.0

500 mM potassium chloride (KCl)

0.5% NP-40

15 mM magnesium chloride ($MgCl_2$) (Thermo Fisher Scientific, cat. no. M33)

Store at 4°C for up to 1 year

Lee's plus N-acetyl-d-glucosamine (GlcNAc) medium—Combine:

950 ml H_2O

17.3 g Lee's powder (see recipe)

0.2 ml 1 mM zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$: Thermo Fisher Scientific, cat. no. Z68)

1.0 ml 1 M $MgCl_2 \cdot 6H_2O$

Adjust pH to 4.0 using concentrated HCl to generate a solution then raise pH to 6.8 using 1 M NaOH.

Add:

20 g agar

1 ml of 1 mg/ml biotin (Acros Organics, cat. no. 230090010; from a 10 mg/ml stock in DMSO, stored at −20°C)

Autoclave 30 min

Cool to 60°C and add:

5 ml of 1% histidine (Sigma-Aldrich, cat. no. H8125)

5 ml of 1% arginine (Sigma-Aldrich, cat. no. A5131)

In a separate vessel, dissolve 12.5 g N-acetyl-D-glucosamine (GlcNAc; Alfa Aesar, cat. no. A1304718) in 50 ml water, filter sterilize, and add to Lee's medium, described above

Store at room temperature for 2 months

Lee's powder

50 g ammonium sulfate $(NH_4)_2SO_4$ (Thermo Fisher Scientific, cat. no. A702)

2 g magnesium sulfate ($MgSO_4$; Thermo Fisher Scientific, cat. no. M65)

25 g dipotassium phosphate (K_2HPO_4; Thermo Fisher Scientific, cat. no. P290)

50 g sodium chloride (NaCl; Thermo Fisher Scientific, cat. no. S271)

5 g L-alanine (Sigma-Aldrich, cat. no. 05129)

13 g L-leucine (Sigma-Aldrich, cat. no. L8000)

10 g L-lysine (Sigma-Aldrich, cat. no. L5626)

1 g L-methionine (Sigma-Aldrich, cat. no. M9625)

0.75 g L-ornithine (Acros Organics, cat. no. 129560250)

5 g L-phenylalanine (Sigma-Aldrich, cat. no. P2126)

5 g L-proline (Sigma-Aldrich, cat. no. 81709)

5 g L-threonine (Sigma-Aldrich, cat. no. T8625)

0.85 g L-arginine (Sigma-Aldrich, cat. no. A5131)

0.5 g L-histidine (Sigma-Aldrich, cat. no. H8125)

Combine. Pulse in blender for five 1-min bursts on “mix” setting.

Store at room temperature for up to 1 year

Lithium acetate (LiOAc)/TE buffer

10 mM Tris·HCl, pH 8.0

1 mM EDTA

100 mM lithium acetate (Acros Organics, cat. no. 447712500)

Store at room temperature for up to 1 year

PEG/TE buffer

10 mM Tris·HCl, pH 8.0

1 mM EDTA

100 mM lithium acetate (Acros Organics, cat. no. 447712500)

40% polyethylene glycol 4000 (Sigma-Aldrich, cat. no. P3640)

Store at room temperature for up to 1 year

Powdered amino acid mix

To prepare ~200 g use:

5 g adenine HCl (Sigma-Aldrich, cat. no. 01840)

10 g L-tryptophan (Sigma-Aldrich, cat. no. T0254)

5 g L-methionine (Sigma-Aldrich, cat. no. M9625)

7.5 g L-tyrosine (Sigma-Aldrich, cat. no. T3754)

7.5 g L-lysine HCl (Sigma-Aldrich, cat. no. L5626)

12 g L-valine (Sigma-Aldrich, cat. no. V0500)

50 g L-threonine (Sigma-Aldrich, cat. no. T8625)

50 g L-serine (Sigma-Aldrich, cat. no. S4500)

Put ingredients in a blender. Use “Mix” setting for five 1-min bursts.

Store at room temperature for up to 1 year

HIS1, LEU2, ARG4, and URA3 are commonly used auxotrophic markers in C. albicans. Thus, this medium is formulated so that one or more of these can be left out for nutritional selection, if required.

US Biological sells a -His, -Leu, -Arg dropout mix (cat. no. D9539–09), which may be more economical for labs requiring smaller amounts of this medium.

Synthetic complete dextrose (SCD) medium and agar plates—To prepare 1 liter, in a 2-liter flask, mix:

850 ml H_2O

20 g agar

7 g yeast nitrogen base (YNB; BD Biosciences, cat. no. B291920)

Autoclave

In a separate beaker mix:

75 ml H_2O

50 ml 40% (w/v) glucose

1.7 g powdered amino acid mix (see recipe; -Ura, -Leu, -His, -Arg)

4.5 ml 1% uracil (Sigma-Aldrich, cat. no. U1128)

10.8 ml 1.25% L-leucine (Sigma-Aldrich, cat. no. L8000)

4.5 ml 1% L-histidine (Sigma-Aldrich, cat. no. H8125)

4.5 ml 1% L-arginine (Sigma-Aldrich, cat. no. A5131)

2.5 ml 1% uridine (Sigma-Aldrich, cat. no. U3750)

Warm slightly to dissolve powdered amino acids and filter sterilize

For SCD agar plates: Cool 850 ml of water/agar/yeast nitrogen base (YNB) mixture to 60°C and add 150 ml of amino acid/glucose mixture to make up 1 liter of SCD medium. Mix thoroughly and pour plates.

Store at room temperature for 2 months

YPD medium—To prepare 1 liter, in a 2-liter Erlenmeyer flask add:

950 ml H_2O

10 g yeast extract (BD Biosciences, cat. no. 212750)

20 g peptone (BD Biosciences, cat. no. 211677)

20 g agar (Thermo Fisher Scientific, cat. no. BP1423)

Autoclave and cool to 60°C

Add 50 ml of 40% (w/v) glucose (Thermo Fisher Scientific, cat. no. D16–3) and 2.5 ml of 1% uridine (Sigma-Aldrich, cat. no. U3750) and pour into petri dishes.

Store at room temperature for 2 months

YPD medium plus nourseothricin—Add 10 ml/liter nourseothricin (clonNAT; WERNER BioAgents) from a 25 mg/ml stock to YPD medium (see recipe).

Store at 4°C for 2 months

COMMENTARY

Background Information

The white-opaque switch has been extensively studied over the past three decades. In particular, interest in this switch was piqued when it was discovered that opaque cells are the mating-competent form of the species (Miller & Johnson, 2002). Mating in *C. albicans* is also regulated by genes encoded at the mating type-like (*MTL*) locus, with most isolates carrying both *MTL***a** and *MTL*α loci (Hull, Raisner, & Johnson, 2000; Lockhart et al., 2002; Magee & Magee, 2000). Mating requires that cells lose information from one of the two *MTL* loci (i.e., become *MTL***a** or *MTL*α) and also switch from the white state to the opaque state (Miller & Johnson, 2002). It was subsequently found that opaque cells of a single mating type can also undergo self-mating under conditions that induce the pheromone signaling pathway (Alby, Schaefer, & Bennett, 2009). As well as regulating sexual reproduction, the white-opaque switch determines infection outcomes, as white cells are more virulent and are effective colonizers of the gastrointestinal (GI) tract whereas opaque cells are more efficient at colonization of the skin (Kvaal, Srikantha, & Soll, 1997; Lachke, Lockhart, Daniels, & Soll, 2003; Pande, Chen, & Noble, 2013). The white-opaque switch is, so far, unique to *C. albicans* and to two close relatives, *C. dubliniensis* and *C. tropicalis*, and in each species cells in the opaque state are the mating-competent form (Porman, Alby, Hirakawa, & Bennett, 2011; Pujol et al., 2004; Xie et al., 2012).

More recently, additional phenotypic states have been identified in *C. albicans* including the gray and the GUT states. The gray state has been observed in clinical strains that undergo tristable white-gray-opaque switching and appears to be formed independent of either Efg1 or Wor1 (Tao et al., 2014). Gray cells were more effective than white or opaque cells at colonization of tongue tissue *ex vivo* (Tao et al., 2014). The GUT state was reported in a subset of cells engineered to overexpress *WOR1* and then recovered following passage through the murine GI tract and these cell types showed increased fitness when re-examined in the GI tract (Pande et al., 2013).

Colony phenotype-based assays provide a simple, reproducible method to determine the white-opaque switching frequencies of different strains or switching in response to changes in environmental conditions. We typically perform these assays on SCD plates followed by colony scoring using a stereomicroscope to examine individual colonies. The appearance of

opaque colonies is subtly different than that of white colonies but can be easily distinguished with practice. Use of either phloxine B or CHROMagar makes these assays easier to score.

Fluorescent fusions and reporter strains making use of genes with state-specific expression patterns is another powerful tool in the study of the white/opaque switch. The transformation protocol described here will be familiar to those studying yeast molecular biology. However, *Candida* is somewhat more difficult to work with as there are no stable episomal vectors currently available and all modifications therefore require integration of constructs into the genome. Comparatively large amounts of transforming DNA (at least 3 μg) are used as integration into the genome occurs with low frequency.

Critical Parameters and Troubleshooting

The relative instability of the opaque state often results in a fraction of cells in an opaque colony switching back to white. This is exacerbated by prolonged incubation on solid medium, so experiments with opaque cells are best done with opaque colonies that have been freshly plated from frozen stocks or using single colonies from a recently sub-cultured plate so that a pure population of opaque cells is obtained. A sizeable proportion of cells frozen in the opaque state are recovered in the white state, so it is therefore critical to confirm the opaqueness of colonies prior to use.

Imperfections and/or debris on the surface of an agar plate can deform a circular colony. Score for the presence of dark sectors with a rougher texture, not deformation. Some mutant backgrounds that affect fitness will show sectoring which may represent spontaneous mutants with a slightly faster growth rate. But these will appear to be white sectors on a white colony and should also not be scored as a white-opaque switching event. A number of mutations that negatively affect fitness have been shown to increase the basal rate of white-to-opaque switching, even though these genes do not regulate the switch *per se* (Alby & Bennett, 2009).

Some mutations and growth conditions can result in alterations of cell shape. *Candida* can grow as pseudohyphae which resemble chains of opaque cells. Mutations which cause transient cell cycle arrest can result in elongated cells (Berman, 2006). Examination of *WOR1* or *OP4* reporters can be used to determine whether an elongated cell is in the opaque state.

Proper sterile technique is critical for a successful switching assay. A stray spore of a filamentous fungus can spread over much of the plate during the 7 day incubation. We typically plate cells in duplicate or triplicate to increase sample size and to provide additional protection against lost data points due to contamination.

Problems with the transformation protocol described herein typically arise either during PCR amplification of the transforming DNA cassette or screening clones for integration at the correct genomic location. Oligonucleotides of the size used here (~100 nt) are sometimes able to form stable secondary structures and are more prone to primer-dimer formation than shorter oligonucleotides. Problematic secondary structures such as hairpins can be eliminated by annealing at a higher temperature. A gradient thermocycler is useful as it

allows testing a range of annealing temperatures simultaneously. We often find that addition of 1.2 M betaine (5 M stock in water, free base, not betaine·HCl) will improve PCR amplification of difficult targets. Primer dimers, which manifest as a wide, intense, low molecular weight (<200 bp) band on an agarose gel, can sometimes be reduced by setting up the reaction on ice then putting the tubes directly into a pre-warmed thermocycler block at 95°C.

Because of the relatively short stretches of homology on either side of the transforming DNA a portion of positive clones will have integrated the selectable marker by non-homologous end joining (NHEJ) rather than homologous recombination. Therefore, colony PCR of the junctions of the integrated DNA is critical. Although integration at the *WOR1* and *EFG1* locus occurs with high frequency (>50%), we have occasionally come across loci that are targeted very inefficiently giving positive junction checks in <5% of transformants.

Anticipated Results

For isolates derived from the standard "laboratory strain" SC5314, switching to opaque is observed in ~5% of white colonies on SCD medium (Zordan et al., 2007). Opaque cells are less stable than white cells, resulting in a higher switching rate from opaque to white. However, other clinical *C. albicans* isolates can undergo white-to-opaque switching at much higher frequencies than SC5314 (Alby & Bennett, 2009; Ramirez-Zavala, Reuss, Park, Ohlsen, & Morschhauser, 2008).

In our hands, transformation of *C. albicans* cells (either using PCR products or cassettes generated by restriction enzyme digests of plasmids) normally result in dozens to several hundred colonies depending on the locus being targeted and the transforming DNA. Colony PCR of eight to twelve colonies for *WH11* and *OP4* reporters or *WOR1* and *EFG1* fluorescent fusions should result in multiple clones with positive junction checks, most of which will give a visible signal by fluorescence microscopy.

Use of Candida-optimized next-generation fluorescent proteins for the study of white-opaque switching—GFP and mCherry have been extensively used in *C. albicans* cell biology since they were first adapted for this organism (Cormack et al., 1997; Keppler-Ross, Noffz, & Dean, 2008). However, in recent years, brighter fluorescent protein variants have become available. mNeonGreen was isolated from the European lancet and is a yellow-shifted green fluorophore which was reported to be several times brighter than GFP (Shaner et al., 2013). mScarlet was generated by mutagenesis of mCherry and was also reported to show a marked improvement in fluorescence intensity (Bindels et al., 2017).

To determine whether these fluorophores outperformed GFP and mCherry in *C. albicans*, the sequences encoding mNeonGreen and mScarlet-I were codon optimized and synthesized, then cloned into pSFS2A to generate pRB895 (pSFS2A-mNeonGreen) and pRB897 (pSFS2A-mScarlet). These plasmids are used as templates to generate cassettes for homologous recombination (as shown in Figure 5) using the DNA overlaps listed in Table 3. Note that the genes were synthesized to encode a GGSG linker which deviates from the design of the GFP and mCherry plasmids described above (pADH76, pADH77). Therefore, different forward oligos must be used with these next-generation fluorescent proteins,

although the same forward oligo can be used for both mNeonGreen and mScarlet. These forward oligos can be paired with the reverse oligos listed in Table 3.

A direct comparison of *C. albicans* Efg1-FP fusions shows that mNeonGreen is ~2.5 times brighter than GFP when imaged using a standard GFP filter set (Zeiss #38) and a Colibri 470 nm LED (Figure 7A, B). Using our Texas Red filter set (Zeiss #45) and a HXP120C halogen lamp, mScarlet shows a modest improvement over mCherry, ~0.6 times brighter. By flow cytometry, both mNeonGreen and mScarlet show a two-fold improvement in fluorescence intensity over GFP and mCherry (Figure 7C).

An unanticipated advantage of the *Candida*-optimized mNeonGreen is that it is much more resistant to photobleaching than the *Candida*-optimized GFP currently in use. In their characterization of mNeonGreen, Shaner et al. (2013) reported that it was brighter than GFP but had similar photostability. We repeatedly imaged fields of cells with 1000 msec exposures (470 nm Colibri LED at 100% intensity) and measured the decrease in fluorescence. Over the course of ten exposures, Efg1-GFP lost almost 90% of its fluorescence whereas Efg1-mNeon lost just over 50% (thus remaining brighter than GFP at its first exposure; Figure 7D). Fitting this data to an exponential curve shows that Efg1-mNeonGreen takes 8.6 sec of exposure under these conditions to lose 50% of its initial fluorescence, whereas Efg1-GFP takes only 3.2 sec to lose the same proportion of its initial fluorescence.

We also tested whether mNeonGreen and mScarlet were an improvement over GFP and mCherry, respectively, in white-opaque reporter assays (Figure 8A). Expressed under the *WH11* promoter, mScarlet is 2.75 times brighter than mCherry, while the *OP4* promoter mNeonGreen is seven times brighter than GFP. Strains expressing both p*WH11* mScarlet and p*OP4* mNeonGreen therefore allow for clear state-specific expression patterns in cells expressing both reporters (Figure 8B). By flow cytometry, populations of white and opaque cells can also be clearly distinguished (Figure 8C). Note that in the analysis of opaque cells, a small proportion of cells had switched to the white state and are now expressing p*WH11*-driven mScarlet. This exemplifies the relative instability of the opaque state. This dual reporter strain also allows us to see state-specific expression patterns at the colony level (Figure 8D). Colonies were grown on SCD at room temperature and images captured using a Zeiss Lumar V12 fluorescence stereomicroscope equipped with a ApoLumar S 1.2× objective and an AxioCam MRmcamera.

Time Considerations

The standard switching experiment described above requires 7 days of incubation but preliminary scoring of colony phenotype is often possible after as little as 4 days (particularly for experiments done on CHROMagar or medium containing phloxine B).

A fluorescent reporter strain can be generated in as little as a week, from PCR amplification of the transforming DNA to confirmation of positive yeast clones by PCR and microscopy. Deletion of the *MTL* locus using restriction enzyme-digested knockout plasmids can be accomplished on a similar time scale.

Acknowledgements

We would like to thank all members of the Bennett and Hernday labs for helping in the development of these assays. This work was supported by National Institutes of Health grants AI141893, AI081704, AI122011, and AI112363 (to R.J.B.), NIH grant AI137975 (to A.D.H), and a PATH award from the Burroughs Wellcome Fund (to R.J.B.).

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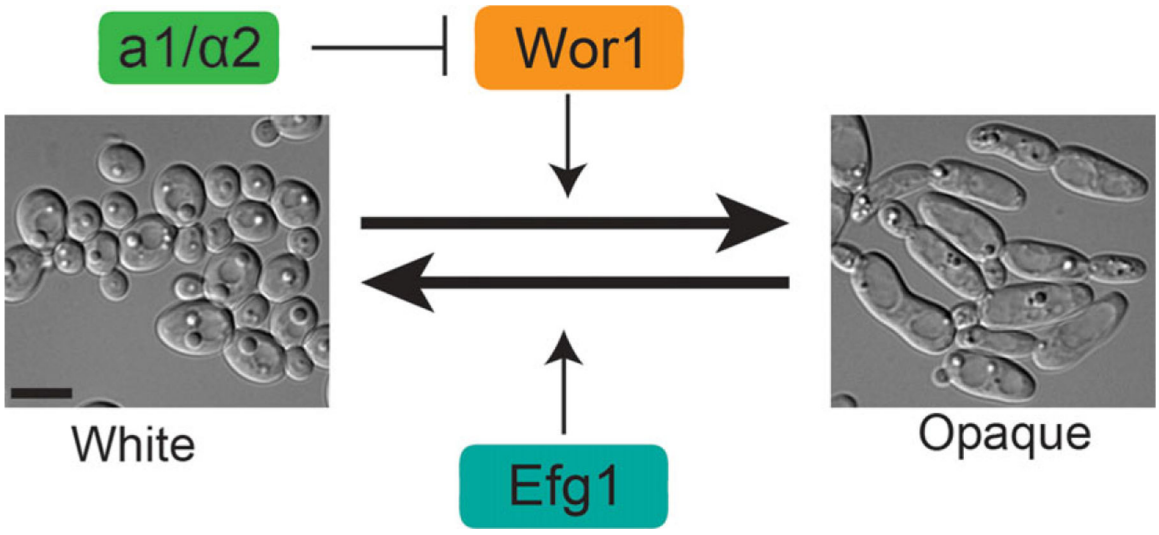

Figure 1.

Regulation of the white-opaque switch. Switching between white and opaque cell states is regulated by a bi-stable toggle switch primarily involving Efg1 and Wor1. Wor1 expression is inhibited by the **a**1/α2 heterodimer, which prevents switching to the opaque state in *MTL***a**/*MTL*α cells. Scale bar, 5 μm.

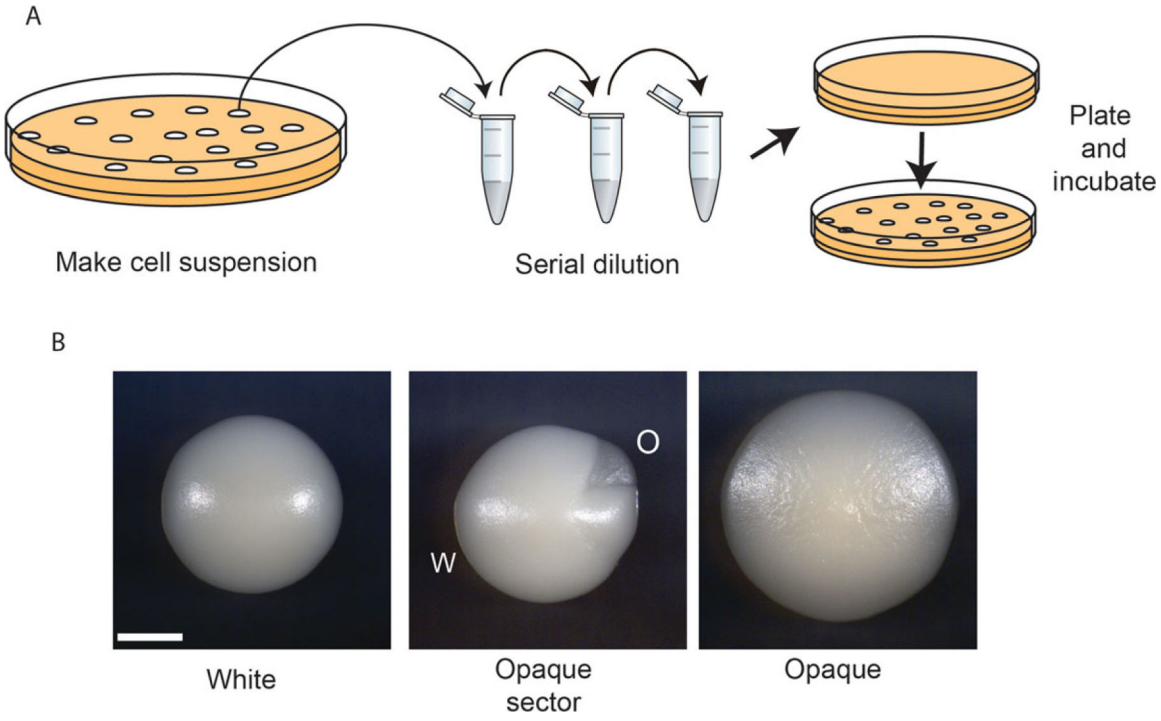

Figure 2.

Basic assay for white-opaque switching. (**A**) Schematic of a standard colony-switching assay. Cells cultured at room temperature are serially diluted, plated at low density, and allowed to form single colonies. (**B**) Cell and colony morphology of white and opaque colonies and the appearance of white-to-opaque sectoring. Cells were plated for single colonies on SCD and incubated at room temperature for 7 days. W, white state; O, opaque state; SCD, synthetic complete dextrose. Scale bar, 1 mm.

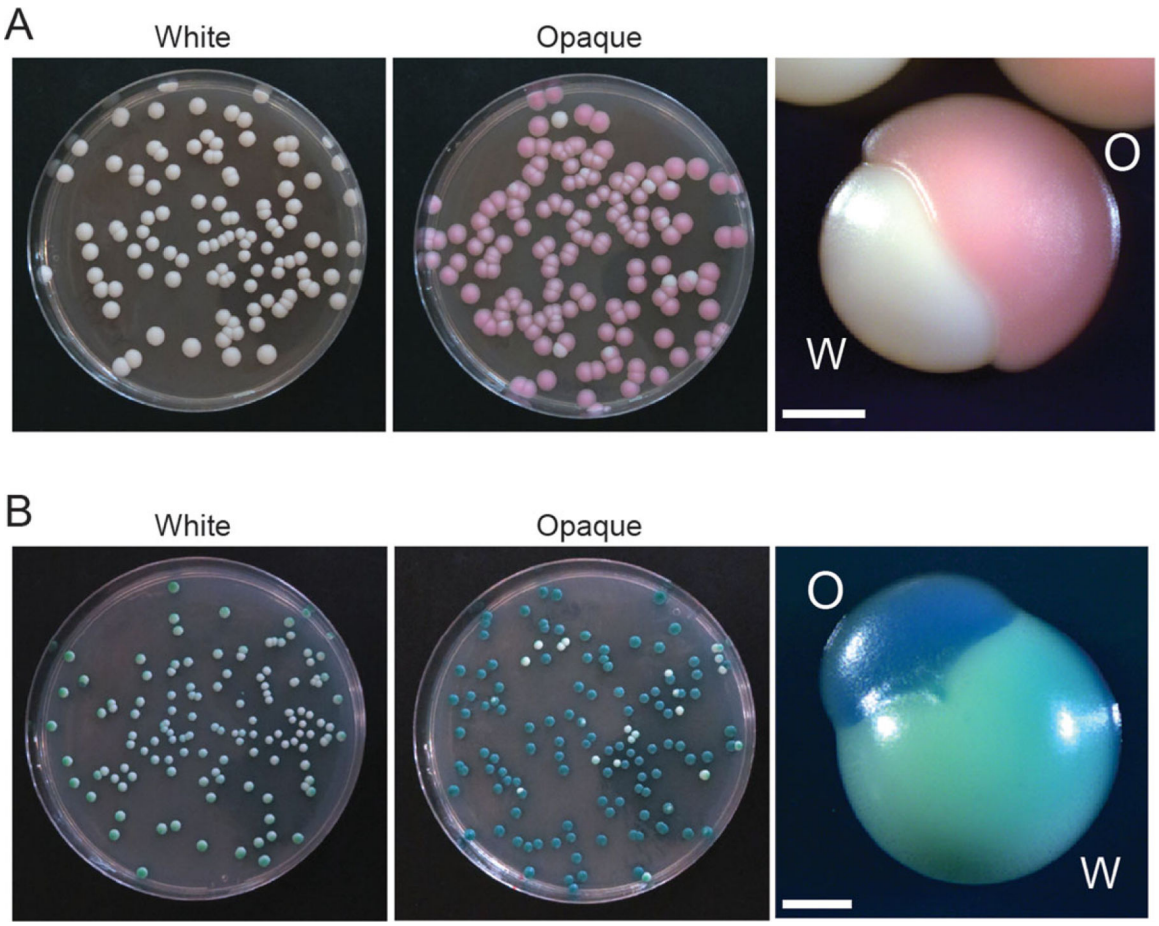

Figure 3.

Alternative media for white-opaque switching assays. (**A**) On YPD + phloxine B opaque cells take up the dye and show pink colonies after incubation for 7 days at room temperature. Scale bar, 2 mm. (**B**) White and opaque colony phenotypes on CHROMagar Candida medium. Scale bar, 1 mm. W, white; O, opaque.

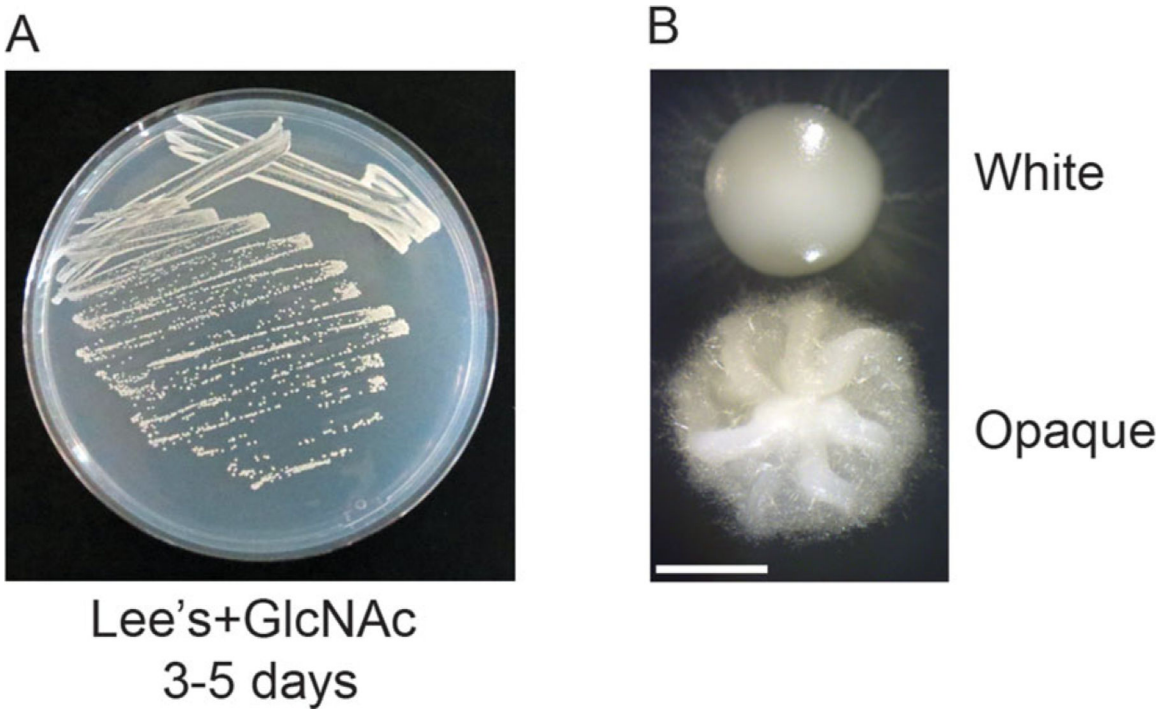

Figure 4.

Isolation of opaque cells from Lee's + N-acetyl-D-glucosamine. (**A**) Cells were densely struck to single colonies and incubated 4 days at room temperature. (**B**) Opaque colonies can be identified by their rough colony morphology, while white colonies are smooth. Scale bar, 10 mm.

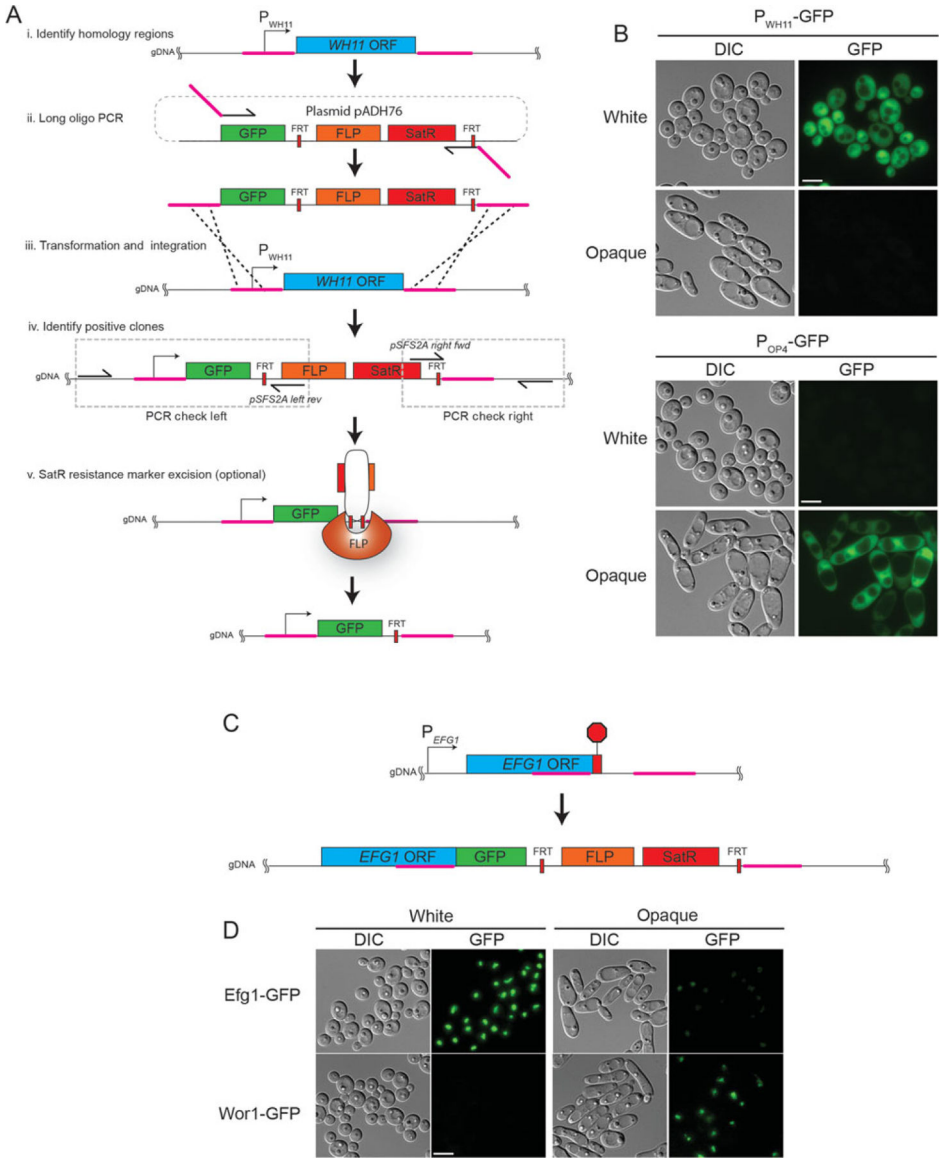

Figure 5.

Generation of fluorescent reporter strains for white- and opaque-specific proteins. (**A**) Primer design, cassette amplification, selection, and analysis for construction of fluorescent reporter strains. i. Identify homology regions: Sequences directly upstream and downstream of the ORF are chosen that will dictate the site of integration for the DNA cassette during transformation. Long oligos are synthesized with ~75 bp of this homology followed by the plasmid sequence overlaps shown in Table 1. ii. Long oligo PCR: A PCR product consisting of the FP and selectable marker is amplified using the long oligos. iii. Transformation and integration: Long oligo PCR product is transformed into a nourseothricin-sensitive *C. albicans* strain and cells that have integrated this resistance marker are selected for. iv. Identify positive clones: PCR across the junction of integration is conducted to show that the transforming DNA has integrated in the correct position in the genome. v. SatR resistance marker excision (optional): *FLP* recombinase expression is induced by culturing on SC

medium containing maltose (instead of glucose) resulting in excision of the SatR marker and the *FLP* gene itself. (**B**) State-specific expression of GFP from the promoter of *WH11* in white cells, and from the *OP4* promoter in opaque cells. Scale bar, 5 μm. (**C**) Generation of transcription factor-fluorescent protein fusions with state-specific expression patterns. (**D**) Efg1 is more highly expressed in white cells than in opaque cells, whereas Wor1 is expressed only in opaque cells. Cells grown overnight in SCD at room temperature. Scale bar, 5 μm. Abbreviations: ORF, open reading frame; FP, fluorescent protein; GFP, green fluorescent protein; SCD, synthetic complete dextrose.

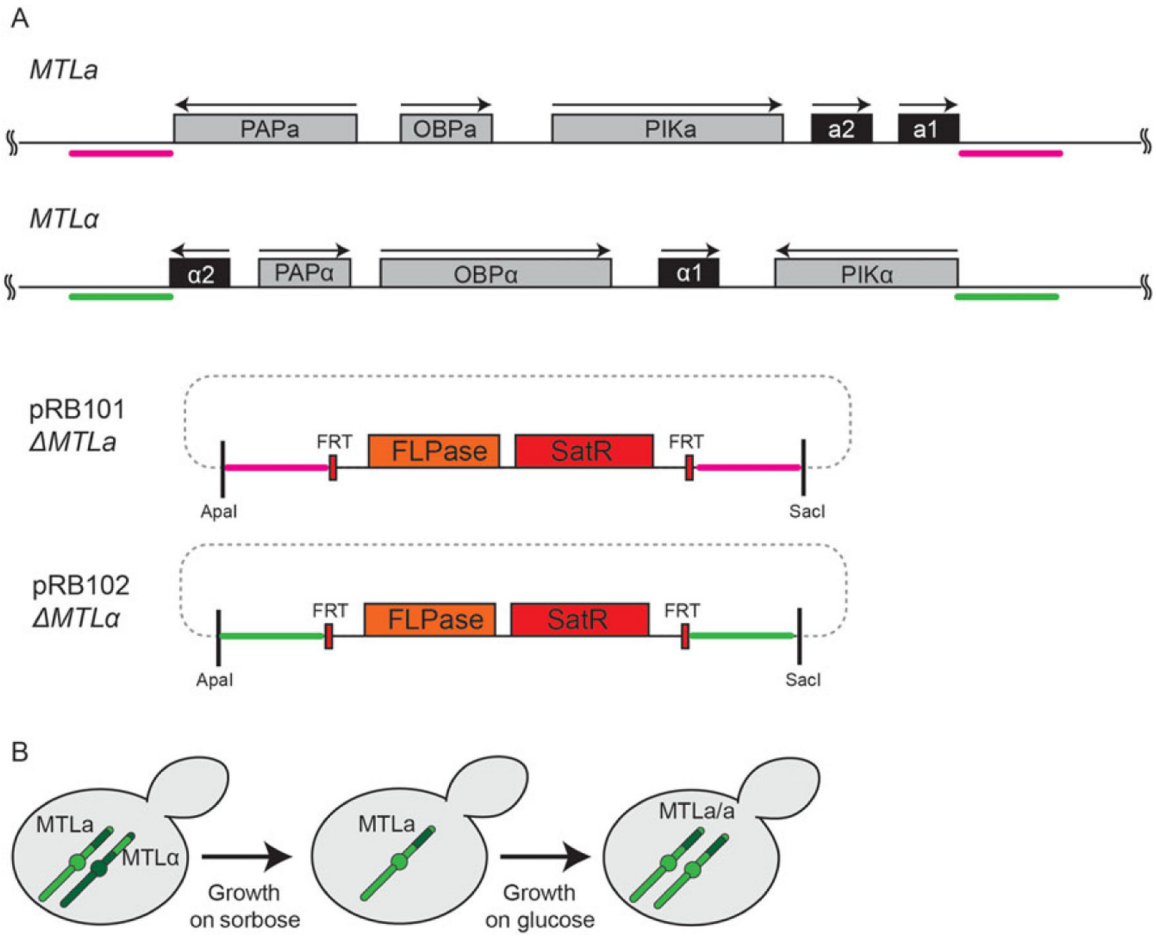

Figure 6.

Generation of *MTL* knockouts for the study of white-opaque switching. (**A**) Schematic of *MTL***a** and *MTL*α loci with regions of homology to the genome indicated. *MTL***a** and *MTL*α disruption plasmids *pRB101* and *pRB102* are shown. (**B**) Generation of *MTL* homozygous strains through selection on sorbose medium and subsequent reduplication of chromosome 5 on glucose-containing medium.

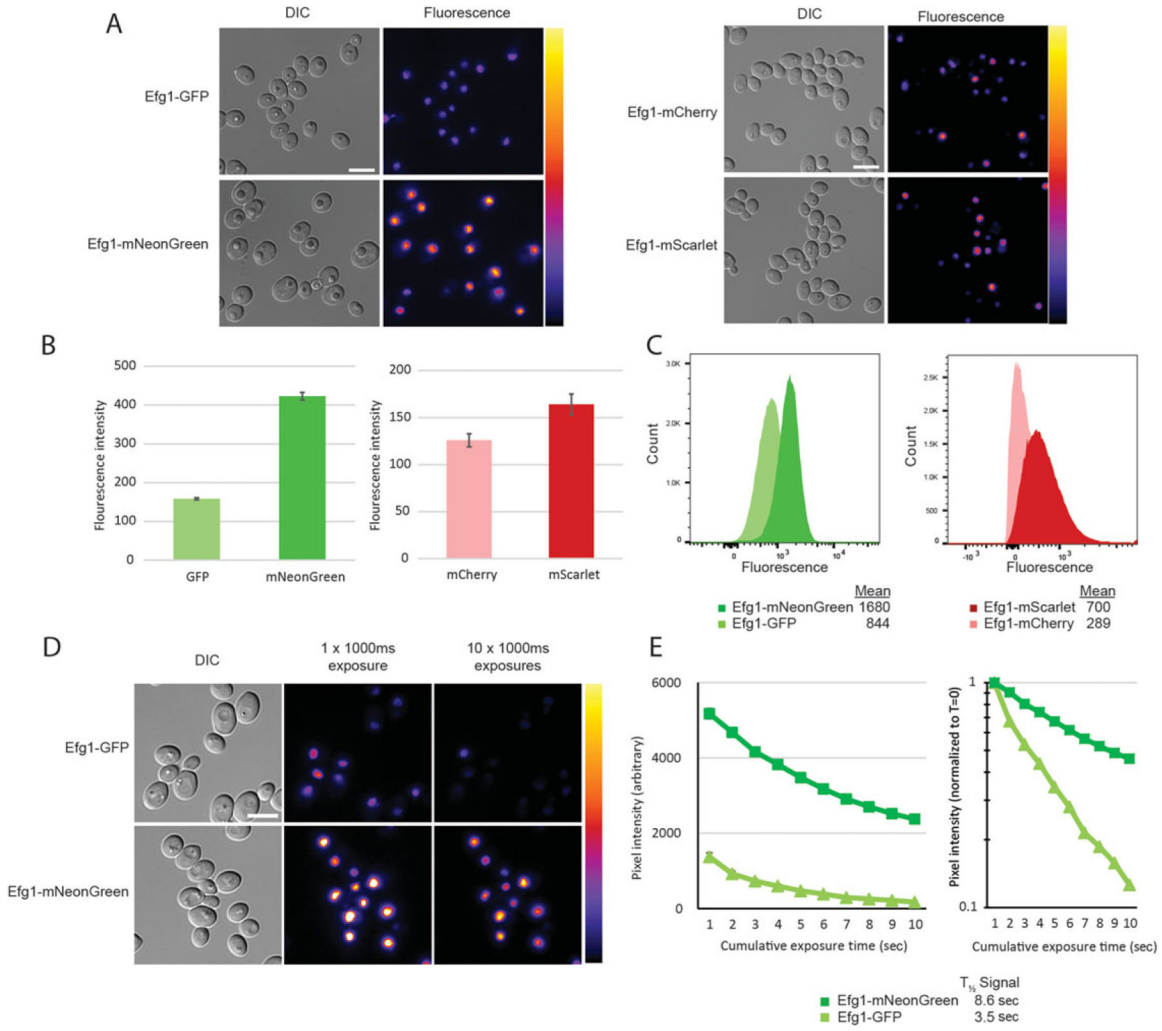

Figure 7.

Microscopy using next-generation fluorescence proteins optimized for *C. albicans*. (**A**) Cell images showing expression of fluorescent fusions of Efg1 with codon-optimized mNeonGreen and mScarlet in comparison to GFP and mCherry. Scale bars, 5 μm. (**B**) Fluorescence intensity of Efg1-mNeonGreen and mScarlet fusions are significantly greater than GFP and mCherry fusions (*t*-test $p < 0.005$). Cells were grown overnight in SCD at room temperature. Average pixel intensity in a circular mask of 15 pixels diameter within the nucleus of at least 90 cells per fusion was measured using ImageJ. Error bars represent standard error. (**C**) Efg1 fusions with mNeonGreen and mScarlet perform better than fusions with GFP and mCherry by flow cytometry. Histograms display fluorescence intensity of 100,000 events. (**D**) Efg1-mNeonGreen is more photostable than Efg1-GFP. Cells were progressively photobleached by repeated 1000 msec exposures at 100% 488-nm LED power. Scale bar, 5 μm. (**E**) Images from the series of exposures from D were transformed into a stack using ImageJ and the average pixel intensity of the same 15-pixel diameter nuclear region was measured for each exposure (n = 10 cells per strain). Data is shown as a decrease in pixel intensity and normalized to T = 0 for each strain. Time to one-half signal was

calculated by fitting the data to an exponential trend line in MS Excel. Abbreviations: GFP, green fluorescent protein; SCD, synthetic complete dextrose.

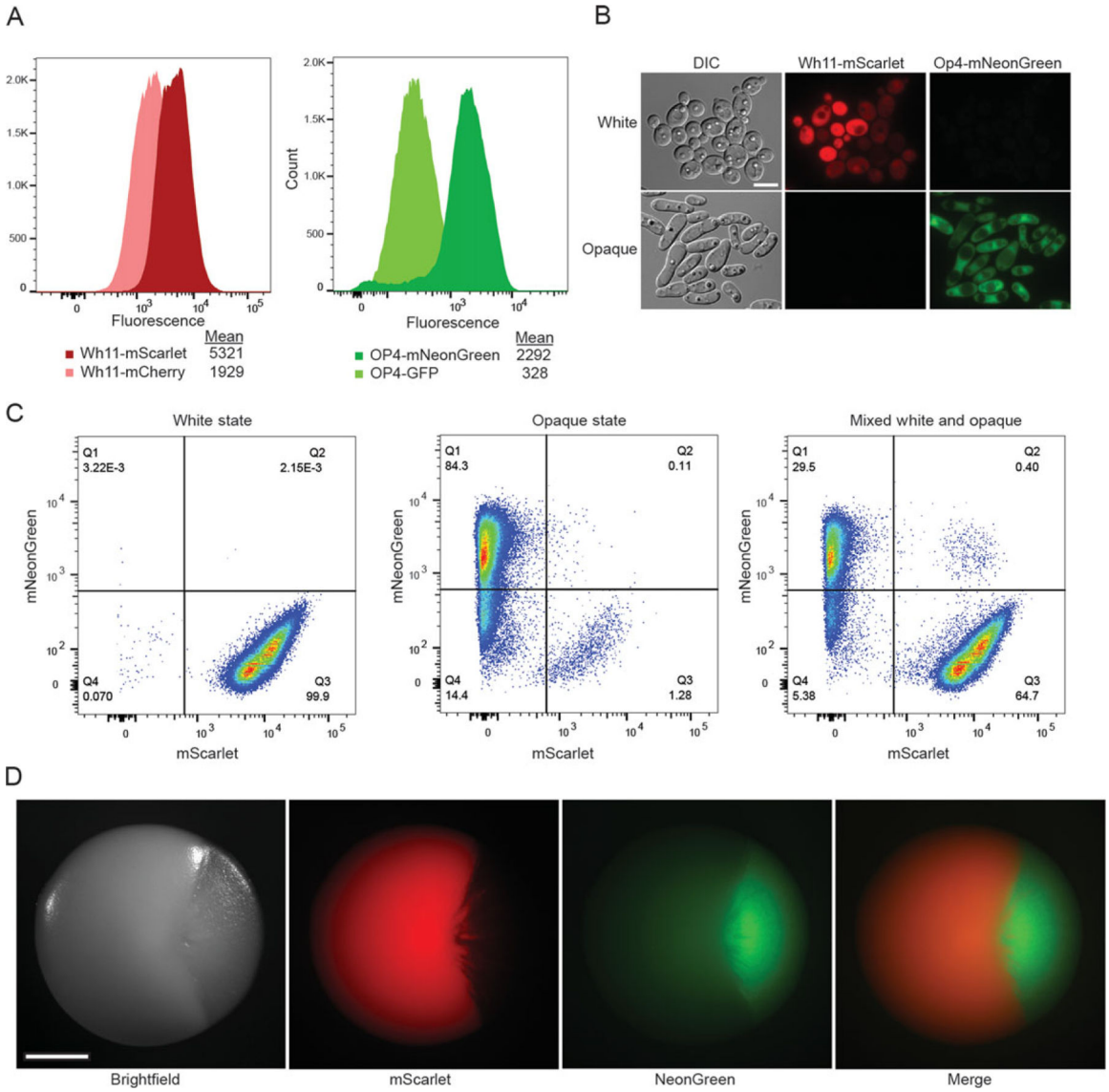

Figure 8.

Flow cytometric analysis of white-opaque reporters using next-generation fluorescent proteins. (**A**) Comparison of mCherry versus mScarlet expressed from the *WH11* promoter and mNeonGreen versus GFP expressed from the *OP4* promoter, as measured by flow cytometry. Fluorescence intensity of 100,000 events per strain. (**B**) Images showing state-specific expression of p*WH11*-driven mScarlet and p*OP4*-driven mNeonGreen in a strain expressing both fluorescent proteins. Cells grown overnight in SCD. Scale bar, 5 μm. (**C**) State-specific expression of p*WH11*-driven mScarlet and p*OP4*-driven mNeonGreen by flow cytometry in white cells, opaque cells, and a mixed white/opaque culture. (**D**) p*WH11*-driven mScarlet and p*OP4*-driven mNeonGreen fluorescence in a sectored colony on SCD. Cells were plated for single colonies and incubated at room temperature for 7 days. Scale bar, 1 mm. GFP, green fluorescent protein; SCD, synthetic complete dextrose.

Table 1

Oligonucleotides for Fluorescent *WH11* and *OP4* Reporters, and for Efg1-FP and Wor1-FP Fusion Proteins[a]

Oligo name	DNA sequence
P_{WH11} forward	5′ - CT TAT TTT CAA TTC ATA TTT CCC TTC CTT TTT TTT TAT CAT TAT AAA ACA AAA CAA CAA ACA GAA CAA TTA AAC ATG ***CGG ATC CCC GGG TTA ATT AAC GG*** - 3′
P_{WH11} reverse	5′ - CA CAC AAA TCT ACA AAT TTG ACA TTA ATT AAT AGC TTC ACA TTT ATT AAA GAA TAT AAA ATA AAC TAA AGA ATA ***GGC GGC CGC TCT AGA ACT AGT GGA TC*** - 3′
P_{OP4} forward	5′ - AT CAA CTA CAA TAA AAC CAA CTA CGT CTA CTA CTA CGT TAA CTC CTA CAC ACA TAC AAA TAT AAA TAA TTT ACA ATG ***CGG ATC CCC GGG TTA ATT AAC GG*** - 3′
P_{OP4} reverse	5′ - AC AGC TTG GAT TAA AGC ACC ACC TTG GAC AAT AGC ACC TTG AAC AGC AGA CTT AAT GAT GTT GGA AAG GGA GCT ***GGC GGC CGC TCT AGA ACT AGT GGA TC*** - 3′
WOR1 forward	5′ - TT ACT AAT GGA ACA TTG GTT GCT GCT GGA ACA GAT GAC GCA GTT GGA AAT TCT T CT GGG TCG TAT TAC ACC GGT ACT [STOP REMOVED] ***CGG ATC CCC GGG TTA ATT AAC GG*** −3′
WOR1 reverse	5′ - CC ATA CCC ACC AGT ATA ATC TGG TTC ATT GAC ATT GTG GTC GTA CTC GTC GTC GGG ACC AAA TTT ACG CTT CTT ***GGC GGC CGC TCT AGA ACT AGT GGA TC*** - 3′
EFG1 forward	5′ - CT CAA GGT TCA GTT CAC CCT TCA CCC CAA CAA CAT CAA GCT AAT CAA TCA GCT AGC ACT GTT GCC AAA GAA GAA AAG [STOP REMOVED] ***CGG ATC CCC GGG TTA ATT AAC GG*** −3′
EFG1 reverse	5′ - TA AAT TTG TCC CAA ATA GTA TAA ATT CGT TCA TGT CAA TGG ATT TGG GAG AAG ATT ATG ATC TAT ACT ATT TCA ***GGC GGC CGC TCT AGA ACT AGT GGA TC*** - 3′

[a]Bold and italicized sequence indicates the region that anneals to the template vector and remains constant; the remainder of the sequence is specific to the gene of interest. Oligos are 100 nucleotides in length. Spacing shows codons of the correct reading frame. We use *pADH76* and *pADH77* as templates for generating GFP and mCherry tagging cassettes, respectively. The start codon of the P_{WH11} and P_{OP4} reporter and the position of the removed STOP codon in Wor1-FP and Efg1-FP forward oligos are indicated.

Table 2

Colony PCR Oligos for pSFS2A Sat1-Flipper-Based Plasmids

Oligo	Sequence
pSFS2A left reverse	5′ - CTC AAC CAT AGC AAT CAT GG - 3′
pSFS2A right forward	5′ - GCG AAA AAG TGG GCA CTA AG - 3′

Table 3

Long Oligo Overlaps for Fluorescent Fusions and Reporter Strains Using pSFS2A-mNeonGreen and pSFS2A-mScarlet Vectors

Oligo	Sequence	Description
mNeonGreen/mScarlet overlap forward	5′ - GGT GGT AGT GGT ATG GTT TCT AAA G - 3′	75 bp of homology to target gene/promoter
Universal overlap reverse	5′ - GGC GGC CGC TCT AGA ACT AGT GGA TC - 3′	74 bp homology downstream of target gene

Table 4

Oligonucleotides for Amplification of *MTL*-Specific Alleles of *OBP* and *PAP* Genes

Oligo	Sequence
OBPα forward	5′ - CCT TCA ATT GCA TCG TAA GTA CC - 3′
OBPα reverse	5′ - GAA GAT GAC TCA GGT CAT GC - 3′
OBPa forward	5′ - GTG GTC AAT GGA GCT GAT AC - 3′
OBPa reverse	5′ - ACA TGT GGT CGC CCA ACT CC - 3′
PAPα forward	5′ - CTG GCA TTC GAT GAA GTC TA - 3′
PAPα reverse	5′ - CAT GTC CGA TTC AAT GGC CC - 3′
PAPa forward	5′ - GGA ATT GAA TTG ATG AAT GAC - 3′
PAPa reverse	5′ - CAG CCC TCT TCC TTT TCG CA - 3′