Chapter 8

Advanced Methods for High-Throughput Microscopy Screening of Genetically Modified Yeast Libraries

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Abstract

High-throughput methodologies have created new opportunities for studying biological phenomena in an unbiased manner. Using automated cell manipulations and microscopy platforms, it is now possible to easily screen entire genomes for genes that affect any cellular process that can be visualized. The onset of these methodologies promises that the near future will bring with it a more comprehensive and richly integrated understanding of complex and dynamic cellular structures and processes. In this review, we describe how to couple systematic genetic tools in the budding yeast *Saccharomyces cerevisiae* alongside robotic visualization systems to attack biological questions. The combination of high-throughput microscopy screens with the powerful, yet simple, yeast model system for studying the eukaryotic cell should pioneer new knowledge in all areas of cell biology.

Key words: Yeast, *Saccharomyces cerevisiae*, Systematic libraries, SGA, Genetic screen, High-throughput, Automated microscopy, Fluorescent marker, Cellular phenotypes

1. Introduction

The field of cell biology was established following the invention of the microscope and the publication of the book "Micrographia" by Robert Hooke in 1665. As microscopes became more advanced, they enabled descriptive works that uncovered constituents of cells and the basic processes that occur within them, such as the cell cycle, division, and death. Two examples for such important observations are the discovery of the Golgi apparatus through microscopic studies of Purkinje cells by Camillo Golgi in 1898 (1, 2) and the first description of chromosomes and their division by Walther Flemming in 1882 (3).

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Over the years, modern cell biology emerged with the attempts to reach a mechanistic understanding of cellular processes using a plethora of molecular, genetic, and biochemical tools. In recent years, such studies have been aided by the emergence of systematic and automated tools for studying the secrets of the cell.

One powerful, yet simple, model system for studying the eukaryotic cell is the budding yeast, *Saccharomyces cerevisiae*. The ease of genetic manipulation in *S. cerevisiae* has enabled the creation of a large number of diverse systematic libraries of either mutated, tagged, or alternatively expressed genes that make up the majority, if not the entire, yeast genome (Table 1). Using sophisticated genetic tricks (4–6), it is now possible to easily create custom-made libraries for monitoring the effect of each gene on a phenomenon of interest. Finally, the combination of these "tailor-made" libraries with the development of automated microscopy and analysis platforms enables high-throughput visual assays to get insights into a broad range of basic processes in cell biology. Such questions range from the fate of a single protein (either its level or localization) through questions of structure and homeostasis of entire organelles and finally questions that address the variability that is found at the population level.

In this chapter, we describe how to easily use these genetic and robotic tools in yeast (Fig. 1) to attack, in an unbiased manner, questions in cell biology that were previously hard to tackle.

2. Materials

2.1. Defining a Biological Question

The updated information on the ever-growing availability of yeast libraries is available at: http://www.openbiosystems.com/GeneExpression/Yeast/.

2.2. Designing a Marker for Screening

2.2.1. Chemical Probes

Chemical probes enable us to detect particular components in living cells, with exquisite sensitivity and selectivity. Today, a large variety of fluorescent chemical probes are available for staining specific subcellular structures or following intracellular conditions, such as viability, redox status, nutrient levels, etc. (http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html).

2.2.2. Genetic Probes

Over the past years, an immense array of genetically encoded fluorescent proteins displaying a wide spectrum of emission and excitation peaks has been described (7, 8). Such a wide selection of fluorophores now enables simultaneous imaging of multiple fusion proteins in living cells. By the use of homologous recombination, these can easily be introduced into yeast to form either fusion proteins or to provide information on activation of promoters.

Table 1
Summary of the major commercially available systematic yeast libraries (in S288C background) important for microscopic screening procedures

Library name and reference	Description	Selection marker
Yeast Knockout Library (27)	This collection includes complete knockouts of the entire open reading frame (from start to stop codon) of all the nonessential yeast genes. Each deletion is also bar-coded. Available in MATa, MATα, and heterozygous diploids	G418 resistance
Yeast DAmP Library (28)	This collection includes hypomorphic alleles for ~82% of essential yeast genes using the decreased abundance by mRNA perturbation (DAmP) method. In short, the 3′ UTR of these genes is disrupted by insertion of an antibiotic resistance marker. The result is a full-length endogenous protein under its natural transcriptional regulation, but at reduced levels	MATa with G418 resistance MATα with nourseothricin resistance
Yeast TS Library (29)	This collection includes temperature-sensitive (TS) alleles of 250 essential yeast genes. Available as MATa	URA+
Yeast Tet-off Promoter Library (30)	This collection includes promoter shutoff strains for ~70% of the essential yeast genes. Created by replacement of the native promoter with a repressible tetracycline (Tet-off)-regulated promoter on the background of a strain containing the Tet inducer cassette at the Ura locus. Available as MATa	URA+ for the Tet inducer locus G418 resistance for the promoter locus
Yeast Gal-GST Library (31)	This collection includes high copy number (2 μ) plasmids that encode for ~80% of yeast ORFs under control of the GAL1/10 promoter (which is activated by growth in 2% galactose) and have an N-terminal GST tag that allows immunoprecipitation assays. Available as MATa	Amp for bacterial resistance URA+ for yeast
Molecular Bar-coded Yeast (MoBY) ORF Library (57)	This collection includes ~80% of yeast ORFs under control of their native promoters and terminator that were cloned into a low copy number (CEN/ARS) plasmid along with two unique DNA bar codes. These plasmids are maintained in bacterial cells	Kan for bacterial resistance URA+ for yeast
Yeast Genomic Tiling Library (58)	This collection contains the yeast genome in 1,588 high copy number (2 μ) plasmids representing a virtually complete overlapping clone collection. The genes are expressed untagged from their endogenous promoters. These plasmids are maintained in bacterial cells	Kan for bacterial resistance LEU+ for yeast

(continued)

Table 1 (continued)

Library name and reference	Description	Selection marker
Yeast GFP Library (32)	This collection includes ~75% of the yeast ORFs tagged at the carboxy terminus with the coding region of green fluorescent protein (*Aequorea victoria* GFP (S65T)). These ORFs are expressed under the control of their native promoter. Available as MATa	HIS+

During the last years, the variety of fluorophores has been enriched by protein molecules that have the ability to act as biological sensors of cellular conditions. This is done by changing the spectral properties of the fluorophores under defined conditions (9). To date, fluorescent-based biosensors have been created to measure cellular redox state, Ca^{2+} concentration, pH, and various trace elements (10–14). By fusing these molecules to specific proteins, it is also possible to target them to specific organelles, thus providing compartment-specific measurements.

2.2.3. Plasmids and Strains

Note: If during creation of a query strain multiple events of targeting are required, it is recommended to use different plasmid families to reduce the possibility of nonspecific recombination between cassettes.

2.3. Creating a Library for Screening

Manipulation of libraries can be performed either manually or robotically. Below are tools that allow either method:

2.3.1. Manual Replication Tools

In order to manually maintain, manipulate, and freeze down libraries, it is best to use a 96, 384, or 1,536 floating pin E-clip style manual replicator alongside grids that enable accurate pinning (all replicators and accessories can be purchased from V & P Scientific, Inc (http://www.vp-scientific.com)).

2.3.2. Robotic Replication Tools

There are a number of robotic systems available that can be programmed to manipulate yeast cell arrays, such as:

Singer RoTor bench top robot (Singer Instruments, http://www.singerinst.co.uk).

VersArray colony arrayer system (BioRad Laboratories, http://www.bio-rad.com).

QBot, QPixXT, MegaPix (Genetix, http://www.genetix.co.uk).

The RoTor is unique in that it uses disposable plastic replicator pads, whereas most other robotic systems use metal pinning tools,

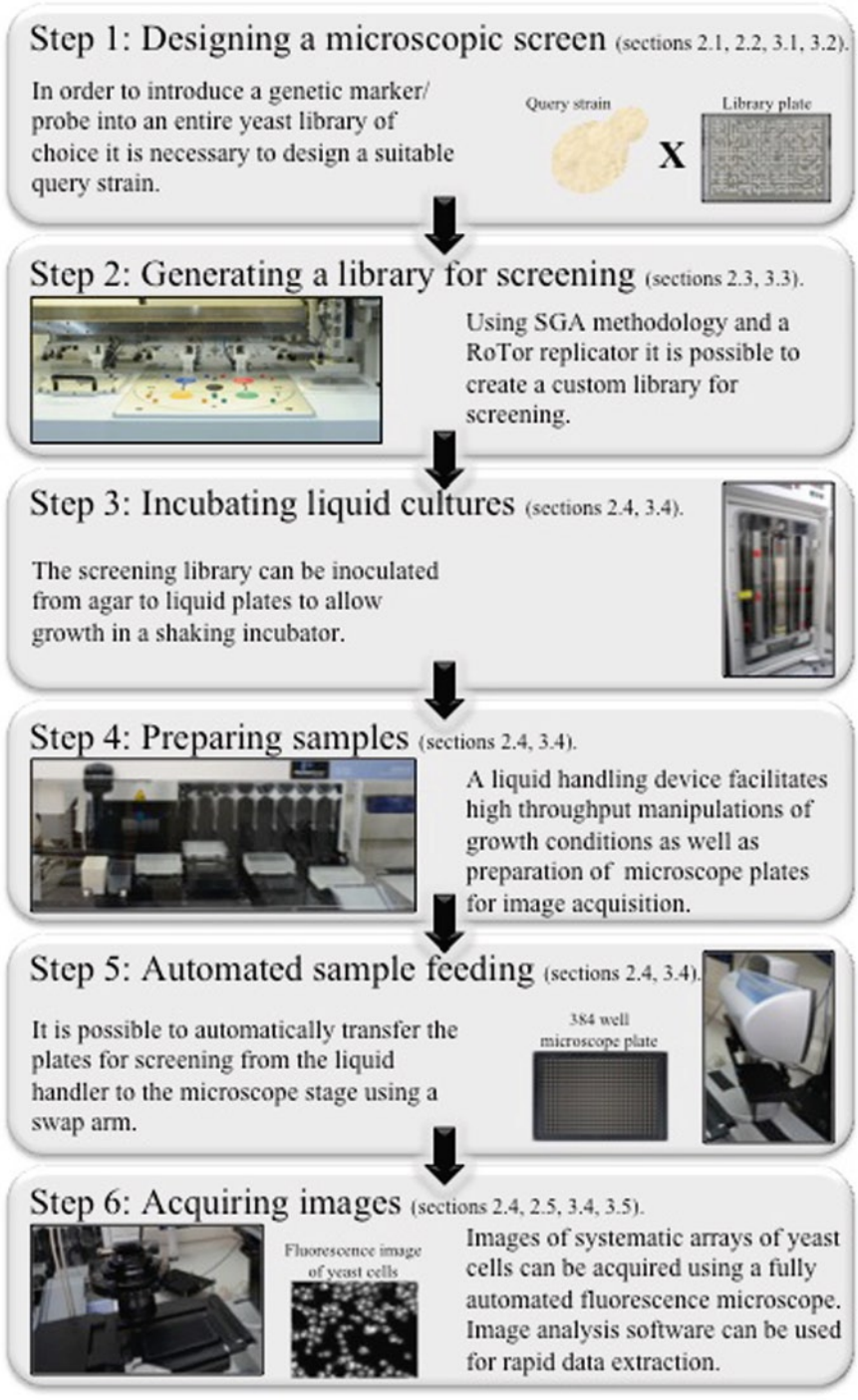

Fig. 1. Schematic representation of the steps required to set up and perform a whole genome microscopic screen in yeast.

which must be sterilized between each pinning step. Disposable plastic increases cost, but allows for higher throughput, since the speed of each pinning reaction is raised dramatically. For a laboratory that routinely maintains and manipulates a large number of libraries, a Singer RoTor is recommended.

Using the Singer RoTor system requires use of specific plates (PlusPlate dishes and lids) and Re-Pads (Singer Instruments).

2.3.3. Performing the SGA

Note: It is impossible to list all media combinations as they depend on the library and query strains used. However, for basic media recipes, see (6, 15).

Materials for Selections

G418 can be purchased from Calbiochem-Merck (http://www.merck-chemicals.com).

Nourseothricin (NAT) from WERNER BioAgents (http://www.webioage.com).

Canavanine and thialysine from Sigma-Aldrich (http://www.sigmaaldrich.com).

Hygromycin from Invitrogen (http://www.invitrogen.com).

2.3.4. Handling Yeast Libraries

Freezing Media

To allow for optimal freezing, use YPD that contains 15% glycerol (glycerol, Anhydrous, J.T. Baker, http://www.jtbaker.nl). However, when freezing libraries that contain plasmids, it is advisable to use the appropriate selection media (which contains 15% glycerol).

Plates and Accessories

Polypropylene plates (PP-MICROPLATE) and lids (PS Lid for Microplate Sterile) can be purchased from Greiner bio-one (http://www.greinerbioone.com).

Sealing foil (AlumaSeal II) can be purchased from EXCEL Scientific (http://www.excelscientific.com).

Library stickers (CRYO TAGS, deep freeze label) can be purchased from GA International (http://www.ga-international.com).

2.4. Running a High-Throughput Microscopic Screen

There are many producers of liquid-handling robots in the market that can be used as automated sample preparation workstations. Below are some examples:

2.4.1. Liquid Handling Platforms

JANUS (PerkinElmer, http://www.perkinelmer.com).

Biomek FXP (BeckmanCoulter, http://www.beckmancoulter.com).

EVO Series (Tecan, http://www.tecan.com).

EpMotion (Eppendorf, http://www.eppendorf.com).

Biorobot Universal System (Qiagen, http://www.qiagen.com).

2.4.2. Imaging Platforms

There are many producers of automated microscopes and high-content screening (HCS) systems in the market that can be used for imaging. Below are some examples:

ScanR (Olympus, http://www.microscopy.olympus.eu).

Cell/Axio Observer (Zeiss, http://www.zeiss.com).

ECLIPSE Ti (Nikon, http://www.nikon.com).

Opera (PerkinElmer, http://www.perkinelmer.com).

Cellomics ArrayScan (Thermo, http://www.thermoscientific.com).

IN Cell Analyzer (GE Healtcare, http://www.gelifesciences.com).

2.4.3. Sample Preparation for the High-Content Microscopy Screen

Growth plates (Polystyrene, PS-MICROPLATE) can be purchased from Greiner bio-one.

Microscope plates (Glass Bottom MicroWell Plate) can be purchased from matrical bioscience (http://www.matrical.com).

Concanavalin A (conA) (C2010-25MG) can be purchased from Sigma-Aldrich.

Paraformaldehyde (PFA) 16% solution can be purchased from Electron Microscopy Sciences (http://www.emsdiasum.com).

KH_2PO_4 – Potassium Phosphate, Monobasic, crystal can be purchased from J.T. Baker.

2.4.4. Image Acquisition

The starting point for any high-throughput microscopic screen is the collection of images for all the strains in the chosen libraries. These images are later used for either manual or automated analysis. Automated microscopes or HCS systems must be purchased/assembled so that they can acquire images with sufficient contrast, resolution, and signal-to-noise ratio to allow image algorithms to extract features of interest. Therefore, when purchasing such a system, focus on several parameters that can significantly impact the quality of the acquired data (16, 17).

1. *Speed*: Speed of the apparatus is important since when scanning large numbers of strains, even small differences in speed may result in large differences in throughput. In addition, for live cell imaging, the time difference between acquisitions of two different fluorophores can cause colocalized structures to move from each other. Parameters that affect the speed are the types of focus that the system employs (hardware or software – see below); motorized items, such as stage, filter wheels, or shutters; communication between the camera and the computer; and camera sensitivity that influences acquisition time.

   Once the system has been purchased, try to maximize the speed for each screen by reducing the number of channels

captured, reducing exposure time, and limiting the number of images that are acquired per well (using 60× magnification may allow acquisition of hundreds of cells per single field. Depending on the type of later analysis (qualitative or quantitative), it may be sufficient to acquire even one position within the well). Another option for increasing the system speed is to use only the software autofocus or the hardware autofocus every tenth well or at the beginning of each row. The better the quality of the bottom of the plate, the less frequently software autofocus must be utilized.

2. *Type of focus*: Most microscopic systems in the market have two integrated focusing options: laser-based (hardware) or image-based (software). In laser-based focusing, the position of a reference point at the interface between the sample and the plate is measured by an external light source. Such autofocus is very rapid and can increase the throughput; however, changes in the thickness of the glass can highly impact focusing making any single reference point insufficient. Image-based focusing relies on acquisition of image stacks followed by selection of the plane for which parameters, such as contrast, resolution, and intensity, decrease on either side monotonically and symmetrically. This process enhances image quality, but increases the scan time dramatically and, if done on a fluorescent channel, can cause bleaching of the respective fluorophore.
3. *Magnification*: To distinguish intracellular compartments and events in yeast cells, a minimum of 60× magnification is required. Most scanning microscopes cannot take images with magnification higher than 60×. This is because most lenses with magnification higher than 60× require a non-air interface to function and this can be hard to achieve with automated screening throughput. To this end, it is most likely that your screen will utilize the 60× lens, so make sure to buy an optimal lens with high numerical aperture (NA).

   Increasing the NA reduces the working distance to the range of 0.17–0.22 mm, making it necessary to work with thin-bottomed plates. Although plastic plates with 0.17 mm thickness could potentially be used, the ones that we tested using our ScanR system could not give images with the same resolution as glass bottom plates.
4. *Compatibility with additional robotic components*: For increasing the throughput, it is recommended to integrate the automated microscopic system with other robotic components, such as liquid handler and automated incubator. This allows automated preparation of the microscopic plates, including incubation, and automated loading of the plates onto the microscopic system.

5. *Camera quality*: In order to acquire high-resolution images while capturing small cellular details, a high-end camera is necessary. Most automated screening microscopes to date already have high-quality, cooled CCD cameras for image acquisition. However, several additional parameters should be considered, such as number of pixels, size of pixels, and quantum efficiency. The number of pixels determines the size of your field, whereas image resolution is determined by the pixel size. The smaller the pixel size, the higher the resolution with optical resolution setting the limit. Thus, pixel size in the range of 6–9 μm is the optimum for 60× magnification. Increasing quantum efficiency increases sensitivity and optimizes the signal-to-noise ratio.

2.5. Analyzing the Data

A critical component of any automated imaging system is the software that serves to control image capture and analysis. Since high-throughput fluorescence microscopy quickly generates data at a much higher rate than can be annotated and evaluated manually, it is advisable to have image analysis tools which couple acquisition with automatic, computerized image processing methods to score the assay and annotate the data in a truly quantitative and unbiased fashion (18).

Regardless of which analysis tool you plan to use, the challenges posed by image analysis are at least as difficult as those faced in image capture. There are multiple levels of image processing: at the pixel level, the object level, the semantic concept level, and the pattern and knowledge level. Images comprise pixels, but it is generally the objects (e.g., cells) represented by the pixels that are of interest. It is, therefore, going from pixel-based representation of data to an object-based representation of data that is the principal challenge in analyzing images, especially in yeast which are small, have buds, and tend to clump together. However, once yeast cells are recognized as objects, it is straightforward for a computer to quantitate their properties, such as shape, size, and fluorescence. The image analysis process involves four basic steps (16, 19).

1. *Preprocessing*: Provides background subtraction and flat-field correction to filter out noise and normalize intensities. Flat-field correction removes artifacts that are caused by small changes in the relative light sensitivity of each pixel in the array of the detector or by nonuniformities in the focal plane. This is performed by compensating computationally for different gains and dark currents in the image.
2. *Segmentation*: Identifies all cells or objects in each image. Depending on the cellular assay, the object of interest could be the whole cell or subcellular structures that are labeled with an appropriate fluorescence marker or captured through the bright-field image. The difficulty of performing segmentation and the algorithms that should be used varies considerably

depending on the cells/objects being segmented and how close they are to each other.

3. *Classification*: Places objects identified as regions of interest into subpopulations of the relevant phenotypic categories. A classification decision can be simple, for example, if it is based on the size or the fluorescence intensity of the object, but it can also be more complex if many parameters or only subtle differences have to be recognized.
4. *Morphological measurements*: Provide quantitative data on important features, enabling production of a unique morphometric profile for the cells being examined. The basic approach is to extract a long list of numerical parameters that describe the shape, texture, and other derived characteristics, such as the pixel intensity statistics, of each object.

Most automated microscope systems come with a commercial software package that allows sophisticated image analysis. Such systems typically provide a number of algorithms to address specific biological applications – it is important to note the range of options provided in each package. It is important to ascertain that the automated image analysis software is intuitive and easy to navigate so that setting and validating the parameters for image segmentation and feature calculations are user-friendly even to biologists with no background in programming. However, for groups that do have expertise in programming, there are packages that allow programming within the main files to allow customization of analysis and increase the options for working with the program. As users become more experienced or require more advanced analysis methods, it is important to ensure that they can easily export files out into more powerful image analysis software packages (16, 17).

3. Methods

3.1. Defining a Biological Question

The availability of fluorescent markers and dyes, coupled with automated microscopy platforms, now allows us to ask an enormous variety of biological questions in a systematic manner (Fig. 1). Literally, anything that can be visualized can be used as a phenotype for genetic screens. Since a large number of systematic libraries exist in yeast, fluorescent markers can be coupled with libraries to create "custom-made" libraries for screening. By and large, three options for combining fluorescent markers with yeast libraries exist.

1. *Combining a fluorescent marker into a mutant library*: Such a process could aid in defining the functional requirements for subcellular processes. More specifically, in order to delineate

the proteins required (directly or indirectly) for the occurrence of a cellular process of choice, it is necessary to create a marker for that process. Processes could be the localization of a single protein (20, 21), physical interactions between proteins (22), the structure of an entire organelle (23, 24), the activation of a single promoter (25), or overall changes in cellular conditions (26). To study the proteins that are required for such cellular processes, these markers should be studied on the background of systematic libraries of mutants, such as the deletion library for nonessential proteins (27), several different libraries allowing downregulation or destabilization of essential proteins (such as the DAmP library (28); temperature-sensitive alleles (29); or the Tet-off collection (30)), as well as the protein overexpression library (31). By screening for the event of choice on the background of all library mutants, we can identify strains displaying an altered phenotype, such as a difference in the expression or localization of our marker, uncovering its biogenesis requirements or regulatory pathways.

2. *Combining a fluorescent marker into a fluorescent library*: This could aid in uncovering novel components of compartments. Although a systematic GFP library has already been created several years ago (32), we still do not know the subcellular localization of hundreds of proteins. These include proteins that could not be tagged or that are not expressed during mid-logarithmic growth in SD medium (the condition in which the GFP library was originally imaged) as well as strains whose tagged proteins exhibit a punctate pattern that could not be colocalized with known compartments of that time. As new subcellular compartments are being discovered (such as P-bodies (33–37); JUNK and IPOD (38); and autophagosomes (39)), their resident proteins can be found by screening the GFP library for colocalization with a query protein.

   For example, to uncover all resident proteins of a new punctate subcellular compartment, it is possible to use a query strain that contains a known marker for this compartment fused to RFP and photograph it in the background of the entire GFP library. A fusion protein with GFP that would colocalize with the RFP marker of the organelle reveals a new protein in this compartment.

3. *Combining a fluorescent marker and a mutation into a mutant library*: This would serve the purpose of understanding the functional relationships and hierarchy of processes within a cell. Specifically, once we have uncovered the basic components of processes, we can also find their functional hierarchy by combining mutations in a single cell (double-mutant analysis) and assaying for their genetic interactions. For this type of question, both a mutation in a gene of choice (such as

a deletion of a specific protein) and a marker protein for our process are studied on the background of systematic mutant libraries. By visualizing these libraries for the functional outcome of this combination, we can identify the cases, where genes are suppressing/buffering or aggravating the phenotype. Such observations would teach us about the structure of the cellular pathways controlling this process (15, 40–47).

Note: Since yeast is infamous for its ability to grow under an endless variety of conditions, it is advisable to perform any such screens under a variety of environments and growth conditions, as the signal may be altered.

3.2. Designing a Marker for Screening

Regardless of which of the three above options you choose to undertake, you first need to consider a way to visualize your process of choice. To do this for automated microscopy, in most cases you would need a fluorescent label (although it is also possible to screen for effects that can be seen by regular bright-field images, such as cell size, shape, cell cycle progress, etc.). There are two ways to create a fluorescent label for your process of choice.

1. *Using a chemical marker/probe*: If a chemical dye exists (Subheading 2.2) to report on the process of choice, then you can decide which library you would like to screen (Table 1) and no genetic manipulations are necessary (if this is the case, skip to Subheading 3.4). Such an approach would save you time; however, it is limited by the number of potential chemical markers and probes available and the high cost of reagents for use on entire libraries.
2. *Using a genetic marker/probe*: The ease of genetic manipulations in yeast alongside the vast array of genetically encoded fluorophores (Table 2 and Subheading 2.2) allows creation of genetic markers for literally any process of choice. For example, by fusing a fluorophore with a protein of choice, it is possible to follow its levels and localization in a living cell under various conditions. By fusing a fluorophore to a compartment-specific protein or by attaching a compartment-localization signal to the fluorophore, any cellular compartment can be labeled. By placing the fluorophore downstream of a transcription regulatory element, it is easy to measure promoter/enhancer activities. Using condition-sensitive fluorophores, readout of cellular/organellar conditions can be achieved. Finally, by using split fluorophores or FRET, it is possible to measure proximity/physical interactions between proteins.

An enormous breakthrough in our ability to use these fluorophores to study cell biological questions in a systematic manner came through the invention of Synthetic Genetic Array (SGA) technology (4–6). This technology allows integration of the marker of choice into only a single query strain (with specific genetic loci

Table 2
Examples of plasmid families for genetically manipulating query strains

Plasmid family and reference	Description
pCG (59)	These plasmids contain auxotrophic selectable markers whose source is from *Candida glabrata* (*Cg*). They can be used for gene deletions in all strains, but are especially useful for manipulating strains that do not have complete deletion of the auxotrophic markers. Include KO plasmids with HIS, LEU, TRP, MET, and URA markers
pFA6 (60, 61)	These plasmids allow for a large number of different genetic manipulations all on the same plasmid backbones: gene deletion, promoter swap, C- or N-terminal protein tagging, and partial N- or C-terminal deletions. The modular nature of these types of plasmids allows efficient and economical use of a small number of PCR primers for a wide variety of gene manipulations. Originally created for use with either antibiotic markers, NAT, Hygro, G418, or auxotrophic markers, HIS, TRP, URA
pYM (62)	These plasmids contain a broad variety of deletion cassettes, C-terminal epitope tags, nine different promoter substitutions (of variable expression levels), and N-terminal tags. The modular nature of these types of plasmids allows efficient and economical use of a small number of PCR primers for a wide variety of gene manipulations. Originally created for use with either antibiotic markers, NAT, Hygro, G418, or auxotrophic markers, HIS, TRP

outlined below), which is easily crossed into any yeast library by the use of pinning tools. This simple method, therefore, allows rapid insertion of any marker into an entire library of choice creating "custom-made" libraries for screening.

3.2.1. The SGA Query Strain

The SGA procedure (outlined in Subheading 3.3) is the method of choice to insert any marker quickly and systematically into entire yeast libraries. In contrast to mass transformation, it does not require liquid handling or affirmation of all strains at the end of the procedure and is fast and robust. In short, this method works by allowing the mating of the query strain to mutant libraries, generating diploids, inducing meiosis to retrieve haploid cells, and finally selecting for only haploid strains that contain the original genetic determinants from both the query strain and the library of choice. To enable this process, an SGA query strain must contain the following genetic features:

Cassette for selection of haploids of a specific mating type: Since mating is an extremely efficient process in yeast, the haploids that are generated at the end of the sporulation process could mate with their "siblings," thereby generating diploids that are heterozygote for both mutation alleles (of the query strain and

the library strains). This would lead to false negatives, as the genetic marker would be imaged on the background of a functional genome. To overcome this problem, the selection of a single mating type following sporulation is required. Therefore, the query strain contains a haploid mating type-specific promoter linked to a selectable marker. Most strains contain a MATa-specific promoter (such as Ste2 or Mfa1) conjugated to the HIS selectable marker and a MATα-specific promoter (such as Ste3) driving the LEU marker.

Loci for selection against diploids: The common library strain (S288C (48)) undergoes sporulation at very low rates (usually, up to 10% of diploids in nitrogen starvation). This leads to very poor chances of finding the desired haploid harboring all the mutations of interest simply by selecting for the markers that are also present in the diploid strain. Moreover, because mitotic recombination can occur between homologous chromosomes in MATa/α diploids, a crossover event between the MAT locus and the centromere on chromosome III can result in MATa/a or MATα/α diploids (25, 49). These types of diploids actually behave like the haploids that were selected for expressing the selectable marker under a mating type-specific promoter, and therefore survive the haploid selection step. To avoid this complication, the SGA strains have two selection cassettes for removal of the diploids following the sporulation step: *can1*Δ and *lyp1*Δ. The CAN1 gene encodes an arginine permease that is permissive also to the toxic analog canavanine. Similarly, the LYP1 gene encodes a lysine permease that enables entry of its toxic analog thialysine (also called S-AEC). Since all library strains are CAN1+ and LYP1+, the heterozygous diploids formed by crossing them against the query strain are all sensitive to these toxic analogs. However, the haploid spores that have the deletions of these two permeases (*can1*Δ and *lyp1*Δ) survive on selection plates that include canavanine and thialysine.

To enable rapid crossing of markers into entire yeast libraries, a query strain should be created. When starting to plan your query strain, consider the following issues:

1. *Suitable genetic background for SGA* (Table 3): Since the first step of the SGA method is mating between a query strain to the library of interest, make sure to choose a strain that has the opposite mating type from the intended library and the right genetic background that is suitable to the specific selections that are going to be used (Tables 1 and 3). For example, a query strain with only a MATa promoter driving LEU (Table 3) should be used for crossing against a library that already harbors a HIS selectable marker (such as the GFP library (Table 1)).
2. *The fluorophore of choice*: A variety of genetically encoded fluorophores are now available (Subheading 2.2). When

Table 3
Examples of existing query strains (in S288C background) for systematically introducing markers into yeast libraries (4–6)

Genotype	Best used to cross against
his3Δ1, leu2Δ0, LYS2+, met15Δ0, ura3Δ0, can1Δ::STE2pr-spHIS5 lyp1Δ::STE3pr-LEU2 **MAT**α	This is the strain of choice for most SGA crosses. It contains two selection alleles against the diploids (Δcan, Δlyp) as well as two options for selecting haploids: a MATa-specific promoter (STE2pr) driving the *Schizosaccharomyces pombe* HIS5 (which is the functional homologue of the *S. cerevisiae* HIS3) and a MATα promoter (STE3pr) driving LEU2
his3Δ1, leu2Δ0, LYS2+, met15Δ0, ura3Δ0, can1Δ::MFA1pr-LEU2 lyp1Δ **MAT**α	This strain should be used to cross against libraries, which harbor the HIS selectable marker (such as the GFP library) since there is no internal HIS selection
his3Δ1, leu2Δ0, LYS2+, met15Δ0, ura3Δ0, cyh2, can1Δ::STE2pr-spHIS5 lyp1Δ::STE3pr-LEU2 **MATa**	This MATa strain can be used to cross against MATα libraries

choosing a fluorophore, it is necessary to consider its basic properties: Is it a monomer or a dimer? How quickly does it fold? Is it very stable? Is its codon optimized for the organism of choice? Does it aggregate at high expression levels? etc. (7, 8, 50). If the screen requires two or more different fluorescent markers, the wavelength overlap of both excitation and emission between these fluorophores must be taken into consideration (Subheading 3.4).

3. *The selection marker*: When transforming the genetic probe into the query strain (whether using a plasmid-based expression system or integration into the genome), a selectable marker must be chosen (Table 2). This selectable marker should be chosen so as not to overlap with the markers for mating type-specific selection (HIS+ for selecting MATa cells or LEU+ for selecting MATα cells) nor with the markers of the destination library. For SGA techniques, the use of positive selection markers (such as Kan^r, Nat^r, or $Hygro^r$) is advisable as they give better selection specificity during the manipulation of the strains and assist in preventing contamination of library plates.
4. *The second marker for an internal control*: The most accurate control is a second fluorophore that reports on the baseline in each cell. This helps assessing variability between cells, wells, plates, and days.
5. *Expression levels of the marker*: It is important that the protein that is chosen as a marker has a high expression level for easy

visualization. This requires either using a highly expressed protein from its endogenous promoter tagged at its C-terminus, N-terminally tagging a protein while changing its promoter to a constitutive and highly expressing one, or utilizing a high copy number plasmid-based expression system (Table 2). High copy number plasmids have variable expression in each cell, so when possible it is preferable not to use them.

6. *The function of tagged proteins*: To ensure that a protein has not lost its function following tagging, compare the tagged strain to a deletion/mutant strain under conditions, where the mutant exhibits a phenotype. If the tag does damage the functionality of the protein and if this is detrimental to the screen, you can insert the marker as a second copy either on a plasmid or at a nonendogenous locus (such as the URA or HO locus), thereby retaining one functional copy.

3.3. Creating a Library for Screening

Once you have created the query strain, you must use SGA technology to introduce your marker of choice into one of the available yeast libraries. Many protocols for performing SGA have been published (for example, (4–6, 15)). To avoid redundancy, we give only some of the basic procedures required to start working with this methodology.

In order to manipulate libraries, it is possible to work with either manual or robotic tools (Subheading 2.3).

3.3.1. Manual Replication Tools

If working with manual tools, it is essential to sterilize them efficiently between each pinning step, as pinning from dense colonies on agar plates causes many cells to remain on the pins, increasing the risk of cross-contamination between plates. Below is a simple, yet efficient, protocol to enable sterilization.

Sterilizing

Wash the pinner sequentially in the following solutions:

Tray 1: Sterile DDW – 1 min or until most of the yeast cells have dropped from the pins.

Tray 2: 10% bleach – 30 s

Trays 3–4: Sterile DDW – rinse

Tray 5: 70% ethanol – 10 s

Tray 6: 95% ethanol – 5 s

Allow excess ethanol to drip off the pins and then place the pinner carefully into the flame of a Bunsen burner (torch) and let it cool before use.

Note: To ensure that the pins are cleaned properly and to avoid contamination in the wash procedure, the volume of wash liquids in the cleaning reservoirs should rise in small increments along the wash steps. However, make sure that all of the pinheads are completely immersed in each solution.

Note: For containers you can use lids of tip box.

Note: The water in tray 1 should be replaced frequently when large amounts of yeast cells can be seen at the bottom of the basin.

Note: If you would like to control for cross-contaminations, press the pinners onto an agar YPD plate following the last daily round of sterilization. If no colonies grow after 2 days, then your sterilization technique is working correctly.

3.3.2. Robotic Replication Tools

Although manual pinning is possible, it is most time-efficient and accurate to use robotic pinning devices (Subheading 2.3). We use the RoToR robotic replicator (Singer Instruments) that utilizes disposable pins. However, all steps are identical for manual replicators or other pinner robots, except for the need to sterilize them between steps. Furthermore, all of the following steps are explained in 1,536 format, but 384 and 96 formats are effective as well.

3.3.3. Performing an SGA (Table 4)

The first steps of the SGA are to prepare a query strain and libraries in a fresh copy of 1,536 format to boost the effectivity of consequent mating.

Arraying the Query Strain and Mutant Library

Day 1: Grow an overnight (ON) culture of the query strain in 20 ml of the appropriate selection medium at 30°C.

Day 2:

1. Pour the query strain culture into a sterile container and use a replicator in order to create a 1,536 array of this culture onto an appropriate selection agar plate. Incubate the plate at 30°C for 1 day.
2. Replicate the relevant libraries in 1,536 format to fresh agar plates with the appropriate selections. Incubate the plate at 30°C for 1 day.

Mating the Query Strain with the Library Array

The purpose of this step is to create heterozygous diploid cells containing the query strain genotype in addition to the library genotype. It is extremely important to mate freshly plated cells. Cells that are incubated for more than 2 days or have been kept at 4°C will mate at a lower efficiency.

Day 3:

1. Replicate the 1,536 array of the query strain onto agar YPD plates.
2. Replicate the library array on top of the query cells.
3. Incubate the mating plates at room temperature (RT) for 1 day.

Note: Following this step, all 1,536 colonies should grow, as there has been no selection pressure.

Table 4
An outline of the SGA routine

Day	Step	Media	Time	Temperature
1	Query strain liquid starter	Liquid media with the specific selection. For example, liquid YPD + NAT for query strain that has nourseothricin resistance	ON	30°C
2	Query strain array	Agar plates with the specific selection. For example, YPD + NAT plates for query strain that has nourseothricin resistance	1 day	30°C
2	Library array	Agar plates with the specific selection. For example, YPD + G418 plates for the deletion library	1 day	30°C
3	Mating	YPD plates	1 day	RT
4	Diploid selection	Plates that contain specific selection to both the query strain markers and the library markers. For example, YPD + G418 + NAT is used for diploid selection of mating between the deletion library and a query strain with nourseothricin resistance	2 days	30°C
5	Sporulation	Nitrogen starvation plates	5 days	22°C/RT
10	Haploid selection	SD-His-Arg-Lys + canavanine + thialysine plates to select for MATa haploids or SD-Leu-Arg-Lys + canavanine + thialysine plates to select for MATα haploids	2 days	30°C
12	Final mutant selection	SD-His/-Leu and -Arg-Lys + canavanine + thialysine + selecting for all markers from both the query strain and the library	2 days	30°C

Diploid Selection

Although mating occurs at high efficiency, haploid cells remain on the YPD mating plates. The goal of this step is, therefore, to select for only the diploid cells that now contain the selectable markers of the query strain in addition to one of the relevant libraries.

Day 4: Replicate the cells from the mating plates onto diploid selection plates that contain the specific selections. For example, if the query strain had a mutation linked to a URA selection marker and it is crossed against the GFP library that has the HIS selection marker, then plate the cells on SD-HIS-URA medium for diploid selection. Incubate the diploid selection plates at 30°C for 2 days.

Note: G418 (for selecting KAN resistance) or hygromycin do not work well on regular SD medium due to the low pH; therefore, if such selection is required alongside an auxotrophic marker, then use synthetic media prepared with monosodium glutamic acid (SD (MSG)) instead of ammonium sulfate (6, 15) to alleviate the acidity of the medium.

Note: Following the diploid selection step, the plates should regain the pattern of the library plate as any "query" colony that had no library strains to mate with would die in this selection. If diploid selection has not eliminated all haploids (as can be seen by the appearance of colonies in places, where no colonies existed in the original library), then it is necessary to go through one additional round of diploid selection. Note that when using two auxotrophic markers, two diploid selection steps should always be performed.

Sporulation

Sporulation is required in order to produce haploid progeny from the selected diploids. Since sporulation is inefficient in S288C cells, it is essential to transfer a large number of diploid cells to the sporulation plates to enlarge the number of spores that are achieved at the end of this process. To increase the percent of sporulation, transfer fresh diploid cells to the sporulation plate (grown for no more than 2 days).

Day 5: Replicate the diploid cells to sporulation plates (nitrogen starvation plates). To transfer a large number of cells, use the "source mix" and "target mix" option in the RoTor or replicate the colonies three times (back and forth) when using manual pinning. Incubate the sporulation plates at 22°C (RT is usually OK) for 5 days.

Note: Make sure to keep the plates humidified and well-aerated. This can be done, for example, by placing them in a cardboard box with a trough of DDW in it. (DO NOT wrap in saran wrap so as to allow maximal airflow.)

Haploid Selection

Due to low sporulation levels, this step eliminates the diploid cells that did not undergo sporulation and selects for haploid spores of a single mating type. These plates are usually SD-HIS-based, if one wishes to select for MATa cells, or SD-LEU-based, if the MATα mating type is required (however, some SGA query strains have different markers, so always make sure to create plates that are tailored to your specific SGA query strain). In addition, to select against the diploids, plates contain canavanine and thialysine and lack arginine and lysine (to avoid allosteric competition on the transporters).

Day 10: Replicate spores onto SD-His-Arg-Lys + canavanine + thialysine plates (Subheading 2.3) to select for MATa haploids or SD-Leu-Arg-Lys + canavanine + thialysine plates to select for MATα haploids. Incubate the haploid selection plates at 30°C for 2 days.

Note: As for the sporulation step also in this haploid selection step, it is essential to transfer a large number of cells from the sporulation plates. For this purpose, use the "source/target mix" options in the RoTor or replicate the colonies three times (back and forth) when using manual pinning.

Note: If haploid spores have a growth retardation phenotype, they may be outgrown by other spores. In this case, consider adding a selection for the mutant cells at the stage of the haploid selection. However, it is advisable not to add antibiotics to this stage as spores may be sensitive to them while germinating even when they contain the resistance cassette.

Final Mutant Selection

The purpose of this step is to finalize the SGA by selecting for the haploid cells of specified mating type that also contains all markers of choice (from the query and the library).

Day 12: Replicate the haploid cells onto SD-His/-Leu and -Arg-Lys + canavanine + thialysine in addition to selecting for all markers from both the query and the library strains. Incubate the haploid selection plates at 30°C for 2 days.

Note: If performing SGA with the TS library, all incubation steps must be done at RT instead of 30°C.

Note: To ensure that the library is completely free of diploids and haploids of the wrong genotype, it is possible to repeat this step.

Quality Control

Once the SGA has been completed, you can ensure that it was successful by selecting several colonies and manually checking that they are indeed haploids carrying both query and library markers. This can be performed by either imaging the cells if two fluorescent markers were selected for or in combination with PCR if one marker is a deletion. In addition, mating-type assays can be performed to verify that the cells are indeed haploids (51). Finally, if your screen includes a positive control, before screening the full library ascertain the validity of the SGA procedure by ensuring visualization of the phenotype in the control strains.

3.3.4. Handling Yeast Libraries

Once a library has been created or purchased, it is very important to handle it carefully in order to keep it in mint condition for long-term use. The agar "working copy" must be refreshed every month by replicating onto fresh agar plates with the appropriate selection. Libraries that have passed the SGA should be kept on the final mutant selection plates to eliminate the chances of remnant diploids overtaking the culture.

It is highly advisable to freeze down the libraries directly after they have been obtained so as to have a pristine stock for future experiments.

Freezing Libraries

1. Replicate the library destined for freezing onto fresh agar plates with the correct selection.
2. Incubate the plates at 30°C for 1 day or until colonies are of intermediate size.

3. Dispense liquid freezing medium (15% glycerol in YPD) to the preferable format of polypropylene plates (usually, 150 µl for 96-well, 50 µl for 384-well, and 10 µl for 1,536-well plates, but may depend on the plate dimensions). This step can be done by employing a liquid-handling robot or manually by using a multipipette. It is important to label plates carefully and descriptively by either writing on the plate or by using special library stickers (Subheading 2.3).
4. Replicate the library strains from the agar plates directly into the polypropylene plates and thoroughly mix the cells to ensure that yeasts are properly suspended. This step can be done using a handheld pinner or the RoTor. Make sure that the plate pattern can be seen at the bottom of the wells.
5. Immediately seal the plates with sterile sealing foil and secure the seal by placing a plastic lid above it. Do not let the yeast sit at RT in the freezing medium for over an hour. Store the plates in a –80°C freezer.

Thawing Libraries

1. Take out the library plates from the –80°C freezer and incubate them at RT or 30°C until completely thawed.
2. Centrifuge the plates for 1 min at 3,000 RCF. The purpose of this step is to spin down drops remaining on the sealing foil in order to avoid cross contamination while opening the seal.
3. Remove the seal and replicate the library strains from the polypropylene plates onto the appropriate agar plates with a handheld pinner or the RoTor. Because the yeast cells tend to sink, it is very important to mix the culture well in the polypropylene plates (with the pinner/RoTor) in order to transfer enough cells to the target agar plate. If this proves difficult, aspirate some of the top liquid following centrifugation to increase cell density.
4. Incubate the agar plates at 30°C for at least 2 days or until all colonies have grown to a significant size.
5. It is possible to reseal the thawed polypropylene plates immediately after use with sealing foil and a plastic lid above it and restore them in the –80°C freezer. Yeast can be frozen/thawed for up to three times. However, efficiency is reduced in every cycle and therefore it is recommended to have one frozen copy that is completely untouched to eliminate the chances of strain loss.

3.4. Running a High-Throughput Microscopic Screen

Once you have created a library for screening (either by use of a genetic or chemical marker), you can proceed to acquire microscopic images. High-throughput microscopic screens enable exploring cell biological changes on a large scale in response to either genetic or environmental perturbations. An

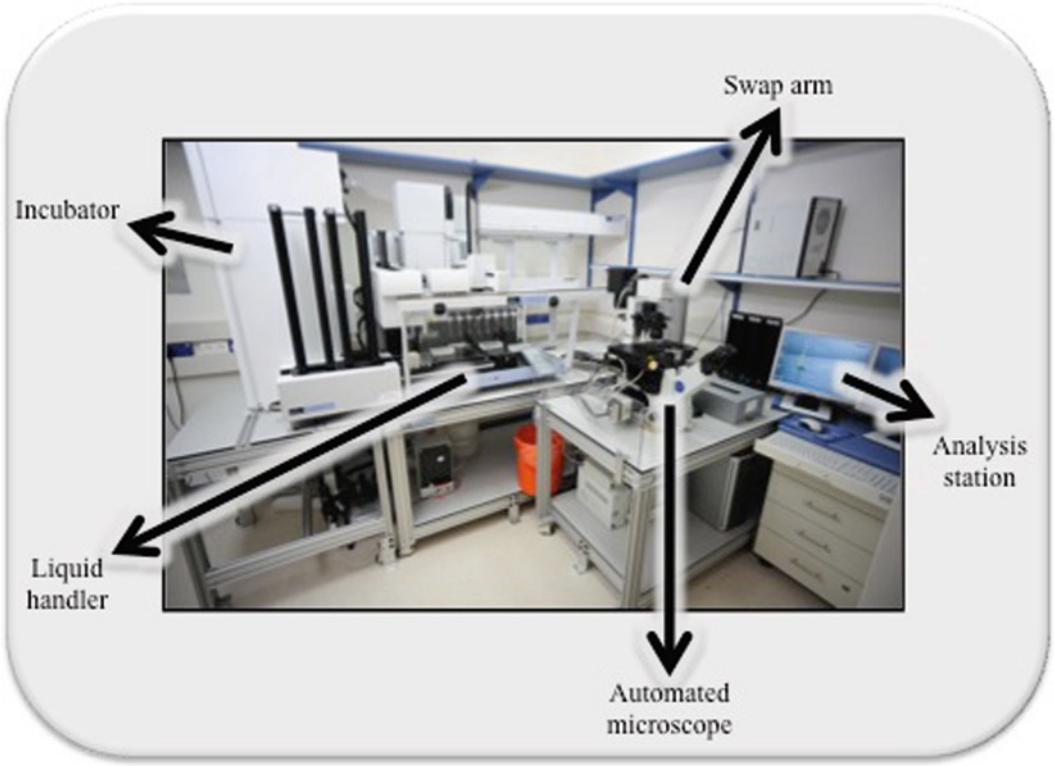

Fig. 2. Example of a setup for high-throughout microscopic screening. Shown is an example of a complete system for microscopic screening. Such a setup can be built in a single room and includes a shaking incubator, a liquid-handing device, a swamp arm, and an automated microscopy platform.

important key for the success of such screens is to use the most suitable automated system. Such a system may include automated sample preparation (liquid handling), automated image acquisition, and automated image analysis for identification of relevant "hits" (16).

Many liquid-handling platforms and automated stage microscopes exist in the market and can be used for such purposes (Subheading 2.4). We have created a fully automated system (Fig. 2) by connecting a shaking incubator (LiCONiC Instruments) through a conveyer belt to a JANUS liquid handler (PerkinElmer), which is connected via a swap robot (Hamilton) to an inverted fluorescent microscopic ScanR system (Olympus) (which brings together both the image acquisition and analysis software). All four stations are controlled via the JANUS Project Manager (JPM) program (PerkinElmer) enabling complete automation of screening procedures allowing us to screen up to 2,000 wells a day. However, good throughput does not require every part of this setup. Below are protocols allowing the screening stages once SGA has been completed.

3.4.1. Sample Preparation for High-Throughput Microscopic Screens

Once the SGA has been completed, the newly formed library is arrayed on agar plates. Before imaging, cells are transferred to liquid media to allow for easy manipulations and for optimal fluorescent signal.

Before transferring your library to liquid format would be a good time to introduce controls. Such controls can be placed within the library in coordinates, where no colonies are growing either before the SGA procedure or onto the final agar or liquid copy. If possible, positive and negative controls (including wild-type cells to measure the baseline or "normal" phenotype) should be placed in EACH plate so that plate-to-plate variability is accounted for. When possible, it is recommended to include an internal control within each cell.

Preparing Liquid Cultures

To ensure optimal image acquisition, prepare liquid cultures in a low-fluorescence medium. Since YPD has very high emission at the wavelengths of fluorophores, such as GFP, it is best to use either SD or the superior specifically formulated screening medium (yeast nitrogen base without riboflavin and folic acid; 5 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.1 g/l NaCl, 0.1 g/l $CaCl_2$, 0.5 mg/l H_3BO_4, 0.04 mg/l $CuSO_4$, 0.1 mg/l KI, 0.2 mg/l $FeCl_3$, 0.4 mg/l $MnSO_4$, 0.2 mg/l Na_2MoO_4, 0.4 mg/l $ZnSO_4$, 2 µg/l biotin, 0.4 mg/l calcium pantothenate, 2 mg/l inositol, 0.4 mg/l niacin, 0.2 mg/l PABA, 0.4 mg/l pyridoxine HCl, 0.4 mg/l thiamine. This medium has negligible autofluorescence – within 10% of water (52)). The microscopic screen can be performed in 96, 384, or 1,536 microscopic plates.

1. Dispense liquid medium to the preferable format of growth plates (150 µl for 96-well, 50 µl for 384-well, and 10 µl for 1,536-well plates; however, the volume can vary depending upon plate dimensions). This step can be done employing a liquid-handling robot or manually by using a multipipette.
2. Replicate the plates for screening from the agar plates into the growth plates. This step can be done using a handheld pinner or the RoTor. Make sure to transfer a small amount of cells (less than 6 OD/ml) so that cells can divide in the liquid medium.
3. Incubate the plates at 30°C ON until cells are in stationary phase.
4. For acquiring images of cells during logarithmic growth, dilute the ON cultures 1:25 into fresh liquid medium.
5. Incubate the plates at 30°C for 3 h.

Note: If any other manipulations are needed (staining, incubation with specific reagents, etc.), they should be done at this stage before transferring the cells onto the microscopic plates.

Note: Library strains differ in growth rates. To ensure that all strains are in mid-logarithmic growth at the time of image acquisition, it is possible to work with plate readers to read ODs and software for calculating and automatically diluting each strain accordingly (53). However, such hardware and software can be complicated to set up and therefore our method gives similar results with less dependence on such tools. The method is based on the assumption that following transfer of cells to media all strains (even ones with long division times) can reach stationary phase if given enough time. To this end, since yeast cultures slow growth dramatically when reaching a certain OD, culturing them ON "synchronizes" the OD of the cultures. The following day, cultures are diluted 1:25 (for example, a WT cell reaches 6 OD/ml which is diluted into 0.24 OD/ml), which ensures that cultures are sparse enough to enable at least two cell divisions after exit from stationary (assuming no culture grows faster than WT) until the diauxic shift (which occurs at 1 OD/ml in WT cells). This ensures that if grown for less than 6 h before acquisition that most cultures will be logarithmically growing.

3.4.2. Preparing Microscope Plates

Concanavalin A Coating of Plates and Transfer of Cells

A monolayer of well-separated cells is best for automated image acquisition and analysis as it easily allows for border detection during segmentation procedures. To achieve optimal adherence, plates should be coated with concanavalin A (conA) (or a similar adherence substance, such as poly-lysine).

1. Wipe the bottom of each microscopy plate with microscopy paper dipped in 100% ethanol to eliminate dust and ensure optimal autofocusing. From hereafter, refrain from touching the glass bottom during manipulations.
2. Dispense conA (0.25 mg/ml in DDW) to the microscopic plates so that the liquid covers the entire base (50 µl for 96-well, 35 µl for 384-well, and 2 µl for 1,536-well plates, but this can vary depending upon the plate dimensions).
3. Incubate the plates for 15 min at RT.
4. Remove the conA by aspiration and leave to completely dry for 30 min.

   Note: Placing plates in a chemical hood reduces drying time to several minutes.

   Note: Plates must be very dry for optimal adherence.

   Note: The residual conA collected from the plates can be recycled for several additional plates.
5. After the plates have completely dried, dispense premixed logarithmic cell cultures (at OD ~0.5/ml) onto the conA-coated microscope plates (150 µl for 96-well, 50 µl for 384-well, and 10 µl for 1,536-well plates, but this can vary depending upon the plate dimensions).

Note: If the cell density is higher than the above OD (for example, if working with stationary-phase cells), then the culture must first be diluted in a separate growth plate. Do not try to dilute cells in the conA-treated plates as this results in multilayers of cells that inhibit the autofocus and reduce image quality following automated acquisition.

6. Incubate at RT for 15 min.
7. Remove the liquid (that contains the cells that did not adhere to the conA) from the plates.
8. Wash the plates with SD medium. Although once is enough, washing three times is recommended for optimal monolayers.
9. Dispense SD (or low-fluorescence medium) to the plates (150 µl for 96-well, 50 µl for 384-well, and 10 µl for 1,536-well plates, but this can vary depending upon the plate dimensions) and visualize.

Fixing Cells

Scanning a multiwell plate at the microscope takes several hours. Exact time depends on the autofocus system used, number of fluorophores imaged, exposure times, and number of pictures that are taken from each well. For two fluorophores with a 1 second exposure time and three images per well, we find that 2 hours are usually required for a 384-well plate. This wide time frame creates a situation, where there might be differences between the acquisition of the first and last wells. Such differences can be detrimental if you are looking at a specific time point after induction of a process or if your process is cell cycle dependant. Moreover, if the conditions used include stress, then it may be alleviated by the time the last wells are scanned.

The solution for such cases is to fix the cells on the microscopic plates before visualization. The fixation of the cells is performed after cells have adhered to the conA plate since fixation reduces the ability of the cells to adhere to conA.

1. Perform the protocol above to adhere cells onto conA-treated plates until step 8.
2. Following one SD wash of the cells (step 8 above), aspirate the medium from the microscopic plates.
3. Add 4% PFA in 4.25% sucrose solution to the cells.
4. Incubate for 15 min at RT.
5. Aspirate the PFA solution and wash the plates with a solution of 0.1 M KH_2PO_4 in 1.2 M sorbitol (KPi solution).
6. Add KPi solution to the plates so as to cover samples (150 µl for 96-well, 50 µl for 384-well, and 10 µl for 1,536-well plates, but can vary depending upon the plate dimensions) and visualize.

3.4.3. Image Acquisition

In order to ensure optimal visualization of cells on the glass-bottomed microscopy plates, several parameters should be considered.

1. *Autofocus*: Most systems have both hardware and software autofocus, which ensure very high focusing accuracy (Subheading 2.4). Software autofocus can be done on bright field or fluorescent images. Use of bright field can result in shifts in focal plane due to differences in diffraction during various growth phases or of cells of various sizes; however, no photobleaching is incurred and the acquisition speed is higher due to lower exposure times. Use of a fluorescent channel requires adding a fluorophore that is distributed evenly in the cells (such as a cytosolic marker) at a high intensity that is different than the fluorophore for quantification of the phenotype since autofocus causes photobleaching; however, it can often be more accurate and also allows for easy image recognition with automated software.
2. *Offset from autofocus*: When looking at intracellular events, the optimal focus may not be the one achieved by autofocusing on the bright field (which usually catches the stack representing either the top or bottom of the cells as they are the most diffractive) or the cytosolic fluorophore. In these cases, in order to optimize image acquisition, calibrating a specific offset from the autofocus is required.
3. *Emission and excitation overlap*: In screens with several fluorescent markers, ensure that emission and excitation spectrums do not overlap in practice. To check if emission overlap exists, simply acquire images of strains expressing only one of the fluorophores in both channels. If no signal is acquired using the second filter, then there is no overlap in emission. Excitation overlap can also be problematic as it may cause bleaching. Since most fluorophores do have some degree of overlap, it is best to utilize narrow excitation filters.
4. *Exposure time*: Most fusion proteins in yeast require between 0.01- and 2 seconds exposure time to be well-visualized. Longer exposure times on living cells may cause blurry signal. This parameter needs to be optimized for each fluorophore before starting large-scale screening.
5. *Number of pictures per well*: This parameter depends on the screening phenotype and the statistics that is needed for ensuring that this phenotype can be measured. It also depends on the density of the cells in the wells. Higher cell density may inhibit the ability of analysis programs to define the edges of cells; however, low-density fields may require multiple pictures which increase the time of the screen and the data load.

   Following calibration of the above parameters, automated screening can commence.

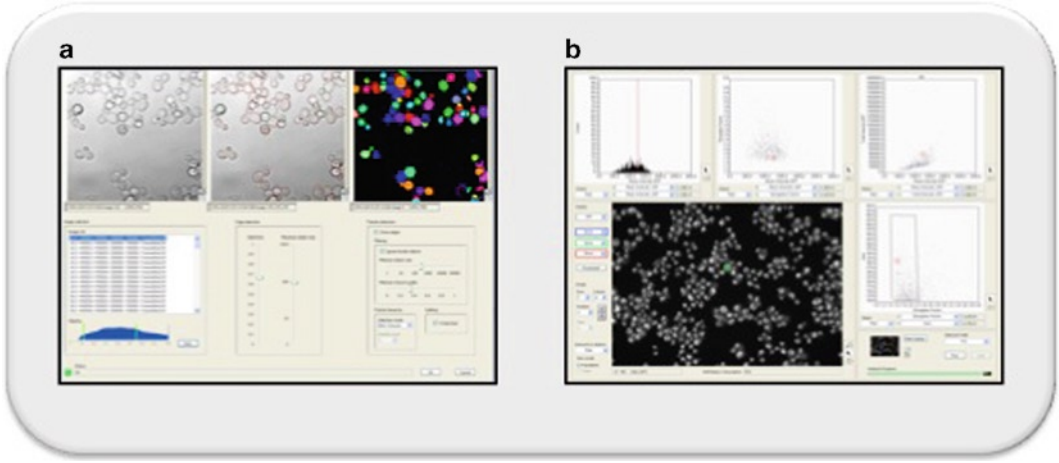

Fig. 3. Example of an image analysis tool. (**a**) Shown is a sample screen from the ScanR program (part of the Olympus ScanR automated microscopy platform). As in any analysis program, the first step of image analysis is segmentation, in which objects are detected within each image. The ScanR edge-based particle detector requires three steps: (1) clipping of unwanted noise or artifacts; (2) edge extraction of the objects; (3) edge closing by combining open edges into single objects. (**b**) Segmentation allows measuring various parameters for each object. Shown are examples of such cutouts for data analysis as either scatter plots or histograms. Gating of specific populations in these graphs can reduce noise and eliminate false measurements. By visualizing the parameters, it is then possible to uncover samples/cells that carry the sought-after phenotype.

3.5. Analyzing the Data

Once images have been acquired, the data must be analyzed to allow identification of strains ("hits") with phenotypes that differ from the control strain. If the screening phenotype is a qualitative one, it is possible to manually assess the images of each strain and score them for the phenotype of choice. However, if a quantitative phenotype is measured, then automation is required. Many screening microscopes come with analysis programs included. We use the ScanR analysis system which enables us to quantify the data automatically in thousands of samples with hundreds of cells in each sample, giving different parameter measurements both at the single cell level and for the whole population (Subheading 2.5 and Fig. 3).

3.6. Confirming Hit Phenotypes

High-throughput screens of systematic libraries are never saturated. This may result from strains that are not present in the libraries, cross contamination of neighboring strains, strains that could not be made during the SGA protocol (for example, two markers that are tightly linked on the chromosome can be hard to create using SGA as they require meiotic recombination to appear together in the final haploid strain), mistakes in the acquisition or analysis, and mutations whose phenotype is covered by a paralogue or a homeostatic response.

Although there is little we can do about such "false negatives" (except for using libraries of different sources and screen twice

using different genetic markers as a phenotype), we can make sure that we do not have "false positives" before starting low-throughput follow-up experiments. To ensure that the "hits" are real and specific to the process of interest, the following should be performed:

1. *Verification of the phenotype*: Strains that were identified as "hits" from the library should be cherry picked into a new plate and rescreened to ascertain reproducibility of the phenotype. This is important in order to rule out differences in phenotype that resulted from variation in liquid handling, cell cycle phase, or strain contamination during the screening procedure.

   Large-scale forward genetic screens often produce a daunting list of genes that impact various biological processes. While such efforts are clearly of great value, they have also overwhelmed our ability to systematically follow up specific biological stories, often resulting in screen outputs comprising long lists of poorly characterized genes. One major problem with such screens is the inability to determine how direct the effect of each mutation is on the phenotype of choice. It is often impossible to determine a direct effect based on the intensity of the phenotype alone since essential genes from hypomorphic libraries have varying levels of downregulation and unessential components often have backup pathways that dampen the observed phenotype.

   Since follow-up experiments often take more time and energy than the initial screening phase, it is important to find ways to focus on the interesting "hits" and weed away uninteresting strains. This can be done by performing secondary screens tailored to the phenotype of interest. Generally, there are two types of possible secondary screens, both to be performed only on the "hit" strains.

2. *Ensuring generalizability of the phenotype*: For this, an additional marker carrying similar properties is assayed in the background of the mutations to assure that the phenotype is reproducible and general.

3. *Verifying specificity*: This screen tests whether other similar processes are not perturbed. For this kind of secondary screen, a marker that can report on the status of additional pathways/routes is integrated and screened.

   Strains that were verified as being both general and specific are good candidates for follow-up experiments. Before proceeding to hypothesis-based experiments, it is best to verify the genotype and its causality.

4. *Verification of the genotype*: In order to ensure that the "hits" harbor the specific mutation that they are thought to contain based on their coordinates in the library, it is essential to check their genotype by check PCR procedure. Another option is to

recreate the specific mutant strains manually and then rescreen. If remaking the strain, it is possible to also use an additional laboratory strain of yeast (for example, W303) to ensure that the phenotype found is general.

Note: It has been shown that many of the deletion library strains contain the selection cassette in the correct integration spot (making them "correct" by most check PCR techniques); however, due to aneuploidy, a normal copy of the deleted gene may still be present in the genome (54). To ensure that the strain with which you are working is indeed deleted for the gene of choice, it is recommended to follow up on the above PCR by using primers from within the ORF. By using such primers, a correct strain would show lack of PCR signal, whereas a product should always be found in a WT strain. To control for lack of DNA or the PCR not working, use control primers, such as actin (for more protocols on check PCR, see (15)).

5. *Verification of causality*: To verify that indeed the mutation that is in the gene of interest is responsible for the phenotype observed (and not a secondary change to promoters/terminators of nearby genes, which is often the case, or a mutation in an overlapping gene on the opposite strand), rescue experiments are essential. This can be done by creating expression plasmids for each hit or more systematically by using either the MoBY library or Gal-GST library (Table 1) as both contain yeast genes on expression plasmids. The plasmids are transformed into the appropriate deletion strains, and this should revert the phenotype back to the control status.

3.7. Creating Genetic Interaction Maps

An alternative to a specific secondary screen is to create a genetic interaction map of the "hits." This is done by systematically combining mutations to form all double-mutant strains carrying the fluorescent marker of choice. To easily perform this, all "hit" strains from the screen (which include both a deletion and the visualization marker) should be crossed against the same deletion strains but of the opposite mating type and harboring a different selection marker. One way to create such a library easily is by taking the deletion library (in either MATa or MATα to have the right mating type) and use a "marker swap" cassette (6, 55) to create a new selection version for these cells.

Previously, such genetic interaction maps have provided concrete information on the function of individual genes and the organization of such genes into pathways and complexes. This suggests that such an effort can dramatically increase the amount and quality of information that can be obtained from a screen while only modestly increasing the amount of time needed to complete it (15, 25, 41, 44–47, 56).

Designing and performing a good genetic screen can pinpoint to novel genes that take part in a cellular process of choice. Performing such screens on entire genomes in a systematic manner has recently become feasible with the development of a large number of yeast libraries, protocols for crossing in markers of choice, and tools to enable rapid microscopic screening. With the increasing ease to generate such data, it is important to use the screening procedure as a stepping-stone rather than a goal. A well-planned and executed screen (and secondary screens) allow for high-quality hypotheses to be generated. In depth follow up on these ideas should allow true detailed and mechanistic understanding of cell biology.

The protocols brought here demonstrate the basic principles of how state-of-the-art tools can be used to tackle fundamental problems in cell biology and as such provide a paradigm for tackling complex biological questions at a variety of organizational levels in eukaryotic cells. By complementing traditional genetic screening approaches with tools for generating custom libraries and systematically visualizing these libraries, this approach can dramatically impact the way cell biology is done today.

4. Notes

All notes can be found under the respective protocols.

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References

1. Farquhar, M. G., and Palade, G. E. (1998) The Golgi apparatus: 100 years of progress and controversy, *Trends Cell Biol 8*, 2–10.
2. Droscher, A. (1998) Camillo Golgi and the discovery of the Golgi apparatus, *Histochem Cell Biol 109*, 425–430.
3. Rieder, C. L., and Khodjakov, A. (2003) Mitosis through the microscope: advances in seeing inside live dividing cells, *Science 300*, 91–96.
4. Tong, A. H. Y., and Boone, C. (2007) High-Throughput Strain Construction and Systematic

Synthetic Lethal Screening in Saccharomyces cerevisiae, in *Methods in Microbiology* (Stansfield, I., and Stark, M. J., Eds.) Second ed., pp 369–386, Elsevier Academic Press.

5. Tong, A. H., Evangelista, M., Parsons, A. B., Xu, H., Bader, G. D., Page, N., Robinson, M., Raghibizadeh, S., Hogue, C. W., Bussey, H., Andrews, B., Tyers, M., and Boone, C. (2001) Systematic genetic analysis with ordered arrays of yeast deletion mutants, *Science 294*, 2364–2368.
6. Tong, A. H., and Boone, C. (2006) Synthetic genetic array analysis in Saccharomyces cerevisiae, *Methods Mol Biol 313*, 171–192.
7. Shaner, N. C., Steinbach, P. A., and Tsien, R. Y. (2005) A guide to choosing fluorescent proteins, *Nat Methods 2*, 905–909.
8. Shaner, N. C., Patterson, G. H., and Davidson, M. W. (2007) Advances in fluorescent protein technology, *J Cell Sci 120*, 4247–4260.
9. Frommer, W. B., Davidson, M. W., and Campbell, R. E. (2009) Genetically encoded biosensors based on engineered fluorescent proteins, *Chem Soc Rev 38*, 2833–2841.
10. Morris, M. C. (2010) Fluorescent biosensors of intracellular targets from genetically encoded reporters to modular polypeptide probes, *Cell Biochem Biophys 56*, 19–37.
11. Ibraheem, A., and Campbell, R. E. (2010) Designs and applications of fluorescent protein-based biosensors, *Curr Opin Chem Biol 14*, 30–36.
12. Wang, H., Nakata, E., and Hamachi, I. (2009) Recent progress in strategies for the creation of protein-based fluorescent biosensors, *Chembiochem 10*, 2560–2577.
13. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin, *Nature 388*, 882–887.
14. Paroutis, P., Touret, N., and Grinstein, S. (2004) The pH of the secretory pathway: measurement, determinants, and regulation, *Physiology (Bethesda) 19*, 207–215.
15. Schuldiner, M., Collins, S. R., Weissman, J. S., and Krogan, N. J. (2006) Quantitative genetic analysis in Saccharomyces cerevisiae using epistatic miniarray profiles (E-MAPs) and its application to chromatin functions, *Methods 40*, 344–352.
16. Vizeacoumar, F. J., Chong, Y., Boone, C., and Andrews, B. J. (2009) A picture is worth a thousand words: genomics to phenomics in the yeast Saccharomyces cerevisiae, *FEBS Lett 583*, 1656–1661.
17. Gough, A. H., and Johnston, P. A. (2007) Requirements, features, and performance of high content screening platforms, *Methods Mol Biol 356*, 41–61.
18. Pepperkok, R., and Ellenberg, J. (2006) High-throughput fluorescence microscopy for systems biology, *Nat Rev Mol Cell Biol 7*, 690–696.
19. Megason, S. G., and Fraser, S. E. (2007) Imaging in systems biology, *Cell 130*, 784–795.
20. Araki, Y., Wu, H., Kitagaki, H., Akao, T., Takagi, H., and Shimoi, H. (2009) Ethanol stress stimulates the Ca^{2+}-mediated calcineurin/Crz1 pathway in Saccharomyces cerevisiae, *J Biosci Bioeng 107*, 1–6.
21. Cai, L., Dalal, C. K., and Elowitz, M. B. (2008) Frequency-modulated nuclear localization bursts coordinate gene regulation, *Nature 455*, 485–490.
22. Lim, R. P., Misra, A., Wu, Z., and Thanabalu, T. (2007) Analysis of conformational changes in WASP using a split YFP, *Biochem Biophys Res Commun 362*, 1085–1089.
23. Anand, V. C., Daboussi, L., Lorenz, T. C., and Payne, G. S. (2009) Genome-wide analysis of AP-3-dependent protein transport in yeast, *Mol Biol Cell 20*, 1592–1604.
24. Kanki, T., Wang, K., Baba, M., Bartholomew, C. R., Lynch-Day, M. A., Du, Z., Geng, J., Mao, K., Yang, Z., Yen, W. L., and Klionsky, D. J. (2009) A genomic screen for yeast mutants defective in selective mitochondria autophagy, *Mol Biol Cell 20*, 4730–4738.
25. Jonikas, M. C., Collins, S. R., Denic, V., Oh, E., Quan, E. M., Schmid, V., Weibezahn, J., Schwappach, B., Walter, P., Weissman, J. S., and Schuldiner, M. (2009) Comprehensive characterization of genes required for protein folding in the endoplasmic reticulum, *Science 323*, 1693–1697.
26. Merksamer, P. I., Trusina, A., and Papa, F. R. (2008) Real-time redox measurements during endoplasmic reticulum stress reveal interlinked protein folding functions, *Cell 135*, 933–947.
27. Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., Arkin, A. P., Astromoff, A., El-Bakkoury, M., Bangham, R., Benito, R., Brachat, S., Campanaro, S., Curtiss, M., Davis, K., Deutschbauer, A., Entian, K. D., Flaherty, P., Foury, F., Garfinkel, D. J., Gerstein, M., Gotte, D., Guldener, U., Hegemann, J. H., Hempel, S., Herman, Z., Jaramillo, D. F., Kelly, D. E., Kelly, S. L., Kotter, P., LaBonte, D., Lamb, D. C., Lan, N., Liang, H., Liao, H., Liu, L., Luo, C., Lussier, M., Mao, R., Menard, P., Ooi, S. L., Revuelta, J. L., Roberts, C. J., Rose, M., Ross-Macdonald, P., Scherens, B., Schimmack, G., Shafer, B., Shoemaker, D. D., Sookhai-Mahadeo, S.,

Storms, R. K., Strathern, J. N., Valle, G., Voet, M., Volckaert, G., Wang, C. Y., Ward, T. R., Wilhelmy, J., Winzeler, E. A., Yang, Y., Yen, G., Youngman, E., Yu, K., Bussey, H., Boeke, J. D., Snyder, M., Philippsen, P., Davis, R. W., and Johnston, M. (2002) Functional profiling of the Saccharomyces cerevisiae genome, *Nature 418*, 387–391.

28. Breslow, D. K., Cameron, D. M., Collins, S. R., Schuldiner, M., Stewart-Ornstein, J., Newman, H. W., Braun, S., Madhani, H. D., Krogan, N. J., and Weissman, J. S. (2008) A comprehensive strategy enabling high-resolution functional analysis of the yeast genome, *Nat Methods 5*, 711–718.
29. Ben-Aroya, S., Coombes, C., Kwok, T., O'Donnell, K. A., Boeke, J. D., and Hieter, P. (2008) Toward a comprehensive temperature-sensitive mutant repository of the essential genes of Saccharomyces cerevisiae, *Mol Cell 30*, 248–258.
30. Mnaimneh, S., Davierwala, A. P., Haynes, J., Moffat, J., Peng, W. T., Zhang, W., Yang, X., Pootoolal, J., Chua, G., Lopez, A., Trochesset, M., Morse, D., Krogan, N. J., Hiley, S. L., Li, Z., Morris, Q., Grigull, J., Mitsakakis, N., Roberts, C. J., Greenblatt, J. F., Boone, C., Kaiser, C. A., Andrews, B. J., and Hughes, T. R. (2004) Exploration of essential gene functions via titratable promoter alleles, *Cell 118*, 31–44.
31. Sopko, R., Huang, D., Preston, N., Chua, G., Papp, B., Kafadar, K., Snyder, M., Oliver, S. G., Cyert, M., Hughes, T. R., Boone, C., and Andrews, B. (2006) Mapping pathways and phenotypes by systematic gene overexpression, *Mol Cell 21*, 319–330.
32. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Global analysis of protein localization in budding yeast, *Nature 425*, 686–691.
33. Tudisca, V., Recouvreux, V., Moreno, S., Boy-Marcotte, E., Jacquet, M., and Portela, P. (2010) Differential localization to cytoplasm, nucleus or P-bodies of yeast PKA subunits under different growth conditions, *Eur J Cell Biol 89*, 339–348.
34. Sheth, U., and Parker, R. (2003) Decapping and decay of messenger RNA occur in cytoplasmic processing bodies, *Science 300*, 805–808.
35. Brengues, M., and Parker, R. (2007) Accumulation of polyadenylated mRNA, Pab1p, eIF4E, and eIF4G with P-bodies in Saccharomyces cerevisiae, *Mol Biol Cell 18*, 2592–2602.
36. Kulkarni, M., Ozgur, S., and Stoecklin, G. (2010) On track with P-bodies, *Biochem Soc Trans 38*, 242–251.
37. Buchan, J. R., Muhlrad, D., and Parker, R. (2008) P bodies promote stress granule assembly in Saccharomyces cerevisiae, *J Cell Biol 183*, 441–455.
38. Kaganovich, D., Kopito, R., and Frydman, J. (2008) Misfolded proteins partition between two distinct quality control compartments, *Nature 454*, 1088–1095.
39. Xie, Z., and Klionsky, D. J. (2007) Autophagosome formation: core machinery and adaptations, *Nat Cell Biol 9*, 1102–1109.
40. Ryan, C., Greene, D., Cagney, G., and Cunningham, P. (2010) Missing value imputation for epistatic MAPs, *BMC Bioinformatics 11*, 197.
41. Fiedler, D., Braberg, H., Mehta, M., Chechik, G., Cagney, G., Mukherjee, P., Silva, A. C., Shales, M., Collins, S. R., van Wageningen, S., Kemmeren, P., Holstege, F. C., Weissman, J. S., Keogh, M. C., Koller, D., Shokat, K. M., and Krogan, N. J. (2009) Functional organization of the S. cerevisiae phosphorylation network, *Cell 136*, 952–963.
42. Dixon, S. J., Costanzo, M., Baryshnikova, A., Andrews, B., and Boone, C. (2009) Systematic mapping of genetic interaction networks, *Annu Rev Genet 43*, 601–625.
43. Boone, C., Bussey, H., and Andrews, B. J. (2007) Exploring genetic interactions and networks with yeast, *Nat Rev Genet 8*, 437–449.
44. Beltrao, P., Cagney, G., and Krogan, N. J. (2010) Quantitative genetic interactions reveal biological modularity, *Cell 141*, 739–745.
45. Schuldiner, M., Collins, S. R., Thompson, N. J., Denic, V., Bhamidipati, A., Punna, T., Ihmels, J., Andrews, B., Boone, C., Greenblatt, J. F., Weissman, J. S., and Krogan, N. J. (2005) Exploration of the function and organization of the yeast early secretory pathway through an epistatic miniarray profile, *Cell 123*, 507–519.
46. Collins, S. R., Miller, K. M., Maas, N. L., Roguev, A., Fillingham, J., Chu, C. S., Schuldiner, M., Gebbia, M., Recht, J., Shales, M., Ding, H., Xu, H., Han, J., Ingvarsdottir, K., Cheng, B., Andrews, B., Boone, C., Berger, S. L., Hieter, P., Zhang, Z., Brown, G. W., Ingles, C. J., Emili, A., Allis, C. D., Toczyski, D. P., Weissman, J. S., Greenblatt, J. F., and Krogan, N. J. (2007) Functional dissection of protein complexes involved in yeast chromosome biology using a genetic interaction map, *Nature 446*, 806–810.
47. Breker, M., and Schuldiner, M. (2009) Explorations in topology-delving underneath the surface of genetic interaction maps, *Mol Biosyst 5*, 1473–1481.
48. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Designer deletion strains derived from

Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications, *Yeast 14*, 115–132.

49. Sychrova, H., and Chevallier, M. R. (1993) Cloning and sequencing of the Saccharomyces cerevisiae gene LYP1 coding for a lysine-specific permease, *Yeast 9*, 771–782.
50. Day, R. N., and Davidson, M. W. (2009) The fluorescent protein palette: tools for cellular imaging, *Chem Soc Rev 38*, 2887–2921.
51. Sprague, G. F., Jr. (2004) Assay of Yeast Mating Reaction, in *Guide to Yeast Genetics and Molecular and Cell Biology* (Guthrie, C., and Fink, G. R., Eds.), pp 77–93, Elsevier Academic Press, San Diego.
52. Sheff, M. A., and Thorn, K. S. (2004) Optimized cassettes for fluorescent protein tagging in Saccharomyces cerevisiae, *Yeast 21*, 661–670.
53. DeLuna, A., Vetsigian, K., Shoresh, N., Hegreness, M., Colon-Gonzalez, M., Chao, S., and Kishony, R. (2008) Exposing the fitness contribution of duplicated genes, *Nat Genet 40*, 676–681.
54. Hughes, T. R., Roberts, C. J., Dai, H., Jones, A. R., Meyer, M. R., Slade, D., Burchard, J., Dow, S., Ward, T. R., Kidd, M. J., Friend, S. H., and Marton, M. J. (2000) Widespread aneuploidy revealed by DNA microarray expression profiling, *Nat Genet 25*, 333–337.
55. Voth, W. P., Jiang, Y. W., and Stillman, D. J. (2003) New 'marker swap' plasmids for converting selectable markers on budding yeast gene disruptions and plasmids, *Yeast 20*, 985–993.
56. Collins, S. R., Schuldiner, M., Krogan, N. J., and Weissman, J. S. (2006) A strategy for extracting and analyzing large-scale quantitative epistatic interaction data, *Genome Biol in press*.
57. Ho, C. H., Magtanong, L., Barker, S. L., Gresham, D., Nishimura, S., Natarajan, P., Koh, J. L., Porter, J., Gray, C. A., Andersen, R. J., Giaever, G., Nislow, C., Andrews, B., Botstein, D., Graham, T. R., Yoshida, M., and Boone, C. (2009) A molecular barcoded yeast ORF library enables mode-of-action analysis of bioactive compounds, *Nat Biotechnol 27*, 369–377.
58. Jones, G. M., Stalker, J., Humphray, S., West, A., Cox, T., Rogers, J., Dunham, I., and Prelich, G. (2008) A systematic library for comprehensive overexpression screens in Saccharomyces cerevisiae, *Nat Methods 5*, 239–241.
59. Kitada, K., Yamaguchi, E., and Arisawa, M. (1995) Cloning of the Candida glabrata TRP1 and HIS3 genes, and construction of their disruptant strains by sequential integrative transformation, *Gene 165*, 203–206.
60. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae, *Yeast 14*, 953–961.
61. Goldstein, A. L., and McCusker, J. H. (1999) Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae, *Yeast 15*, 1541–1553.
62. Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., and Knop, M. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes, *Yeast 21*, 947–962.