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Minireview

A picture is worth a thousand words: Genomics to phenomics in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

Large scale cell biological experiments are beginning to be applied as a systems-level approach to decipher mechanisms that govern cellular function in health and disease. The use of automated microscopes combined with digital imaging, machine learning and other analytical tools has enabled high-content screening (HCS) in a variety of experimental systems. Successful HCS screens demand careful attention to assay development, data acquisition methods and available genomic tools. In this minireview, we highlight developments in this field pertaining to yeast cell biology and discuss how we have combined HCS with methods for automated yeast genetics (synthetic genetic array (SGA) analysis) to enable systematic analysis of cell biological phenotypes in a variety of genetic backgrounds.

1. Introduction

A primary goal of functional genomic projects is to reach a complete understanding of cellular gene function and biological pathways in molecular detail. The budding yeast, a simple and genetically tractable eukaryotic system, is a premier model organism for functional genomic work, and has been used in many pioneering efforts, such as the systematic construction and analysis of gene deletion mutants [1]. Although incredibly powerful, functional genomic approaches that explore gene expression [2–5], protein–protein interactions [6–10] and genetic interactions [11,12], fail to yield a spatio-temporal resolution that will be required to understand biological process as complex dynamic systems. Quantitative cellular imaging techniques can provide this information and produce a wealth of data suitable for thorough statistical evaluation, a key component in systems biology. In this mini-review, we consider progress in applying quantitative imaging to the systematic exploration of gene function and biological pathways in budding yeast.

Immunofluorescence microscopy has been widely applied in cell biology since its development by Coons and Kaplan in the 1950s. More recently, image-based assays for early-stage drug discovery have fuelled the demand to create and refine methods for high-throughput image acquisition and analysis. The term high-content screening (HCS) was first coined in the late 1990s [13] and HCS imaging methods have been applied to a number of different studies in mammalian cells, including drug/small molecule target identification [14–18], screens with G-protein-coupled receptors [19–21] and the RNAi-based analysis of genes affecting a number of different cellular functions, including cell morphology, cell cycle progression, mitosis, cell viability and endocytosis [22–28]. Gene silencing or knockdown in mammalian cells relies largely on RNAi technology, which often creates a hypermorphic rather than deletion phenotype and can exhibit significant off-target effects [29,30]. In addition, many large-scale phenotypic analyses are based on experimental readouts from fixed cells that are subject to multi-step staining techniques, which are not easily adaptable to high-throughput screening [31].

Flexibility in growth conditions and genetic manipulability, coupled with a shorter life cycle, make yeast an excellent system to develop and apply live-cell HCS technology. The ability to perform systematic image-based screens of the yeast proteome has been enabled by the creation of important functional genomic reagents and techniques. The yeast deletion collection, which contains the set of ~5000 viable *KanMX*-marked deletion mutants, and other mutant collections have been assayed for a number of quantitative morphological phenotypes (cell shape, actin cytoskeleton, nuclear morphology and cell size [32–34]). Collections of yeast strains have also been constructed in which each ORF is fused with affinity or fluorescence tags, allowing the first comprehensive view of an expressed eukaryotic proteome and its subcellular localization [35,36]. The collection of 4156 strains with individual GFP-ORF fusions has been used to survey the subcellular localization of

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much of the yeast proteome, using standard fluorescence microscopy. This analysis revealed correlations between protein localization and mRNA co-expression and between protein–protein interactions and subcellular co-localization, emphasizing the power of combining large-scale datasets to deduce physiological relevance. HCS technology promises to enhance the power of analyses using GFP-tagged collections since it allows for acquisition of multi-dimensional phenotypic profiles of a single cell in response to different environmental and drug stimuli (see below for more discussion).

2. Assay development

HCS aims to evaluate cell biological changes on a large scale in response to either genetic or environmental perturbation. As with any useful screen, the development of a robust assay is key for success. For example, we have developed a platform for high-throughput imaging of yeast cells that enables systematic analysis of the cell biological consequences of gene deletion [37]. The main components of our assay are similar to those developed for other HC screens and include: (a) automated sample preparation (including genetic manipulation); (b) automated image acquisition; (c) automated image analysis and extraction of key features; and (d) data mining and identification of biologically relevant hits. An overview of our assay protocol is shown in Fig. 1. We note that, with slight modifications, our methodology can be used in other assays such as image-based chemical profiling, a common application with higher eukaryotic cells.

2.1. Automated sample preparation

Our HCS assay was designed to explore yeast cell biology by assessing loss-of-function phenotypes for a variety of cell biological markers using the yeast deletion collection. A key challenge is to efficiently incorporate the cell biological marker of interest into the deletion collection, which is comprised of ~5000 individual yeast strains. To address this challenge, we make use of synthetic genetic array (SGA) methodology, which allows marked genetic elements to be combined in a single haploid cell through standard yeast mating and meiotic recombination via an automated procedure [12,38]. Alternatively, stains or antibodies can be used to visualize a particular subcellular compartment. For example, Ohya et al. fixed and triple-stained each strain in the deletion mutant collection to visualize cell wall, actin and the nucleus while Wheeler and Fink used antibodies to identify cell wall proteins [33,39].

For live cell imaging, we use robotic pinners to make arrays of liquid yeast cultures by inoculating either 96-well or 384-well format plates from agar plates with the arrayed deletion collection. Slight variations in growth rate cause significant changes in cell density, resulting in some images with too many or too few cells. To ensure uniform and optimal cell densities for subsequent image analysis, we employ a liquid handling robot (Biomek FX), to dispense appropriate volume of samples based on the cell density. Cells are re-suspended in a low fluorescence medium [40] in 96- or 384-well optical plates, prior to imaging (see below).

Other methods for preparing yeast cells for live imaging on large scale have been developed. Kohlwein and coworkers used confocal microscopy to image live cells on agar pads to study lipid metabolism [41]. Marcotte and colleagues developed a spotted high density cell microarray system to detect aberrant morphological changes and to monitor the dynamic localization of proteins at the bud tip in response to mating pheromone [28,42]. The cell arrays are made by contact deposition of suspension cells from an arrayed library onto a single glass slide using a microarray robot. Replicate chips can be made using a spotter and treated with different antibodies to reveal changes in protein localization across the whole deletion collection. Automation of large scale sample processing steps ensures consistency and efficient processing with increased throughput. Several integrated systems, for example BioCell from Velocity 11 (Agilent Technologies) or MaX WorkCell (Thermo Fisher Scientific Inc.), provide complete automated platforms for sample preparation.

2.2. Automated image acquisition

The key component of any large-scale cell biological screening system is an automated microscope. Several vendors offer a variety of either wide-field HCS imagers or confocal imagers and a number of systems are listed by Gough and Johnston [43]. However, to track intracellular events in yeast cells, a minimum of 60× magnification would be ideal. Important system features include speed, compatibility with other components, magnification and the type of focus. Speed is limited by several factors, namely the number of channels, the exposure time and the number of images per well. The choice of laser-based or image-based focusing also influences screening speed, since it affects the rate of scanning a plate. In laser-based focusing, an external light source (typically a laser or laser diode) measures the position of a reference point at the interface between the sample and plate. Although this auto focus can increase the throughput, a single reference point may not be sufficient and may render the sample out of focus. On the other hand, image-based focusing achieves fine focus by imaging several planes and selecting a particular plane from which quantitative parameters such as contrast, resolution and intensity decrease on either side monotonically and symmetrically. This process invariably increases the scan time. Automated focussing in general is time-consuming, so minimizing the focal range using plates with small bottom variations can substantially increase throughput. Magnification is yet another important criterion that affects screening throughput as a higher resolution will capture a smaller field view with fewer cells. Yeast cells are much smaller than mammalian cells (5–7 μm versus 15–25 μm diameter) and with a 60× objective, imaging at least 4–6 sites per well, one can gather as many as 200 cell images. However, depending on the specific biological requirement of the assay, a 20× or a 40× objective may serve the purpose [39,44].

The introduction of the Nipkow spinning disk in some recent machines [Opera (Evotech, Perkin Elmer); Pathway-HT (BD Bioscience)] aids in near real-time image capture with high resolution. Although confocal systems offer higher resolution than standard fluorescence microscopes, their cost and complexity are not required for many primary screens. In addition, illumination can be hindered by the spinning disk, necessitating longer exposure times [45]. We developed our protocol using both a wide-field (ImageXpress 5000A, Molecular Devices) and a confocal system (Opera). We found that the ImageXpress 5000A can image a two channel 96-well plate with 4 images per well in about ~45 min while, in the same time, the Opera can image a 384-well plate with the same specifications. Gough and Johnston provide a comprehensive review with detailed descriptions of various HCS system requirements [43].

To increase our throughput, we have linked Cytomat automated incubators (Thermo Fisher Scientific Inc.) to the ImageXpress 5000A system to allow automated loading of plates into the imager using a CRS Catalyst Express robotic arm (Thermo Electron Corporation) and integration software [Polara (Thermo Electron Corporation)]. Generally two types of automation can be used to control the integration software: dynamic and static protocols. A dynamic approach processes the plates using a specified protocol as the instrument becomes available, increasing the throughput of the screen. However, this approach causes variation in how each plate

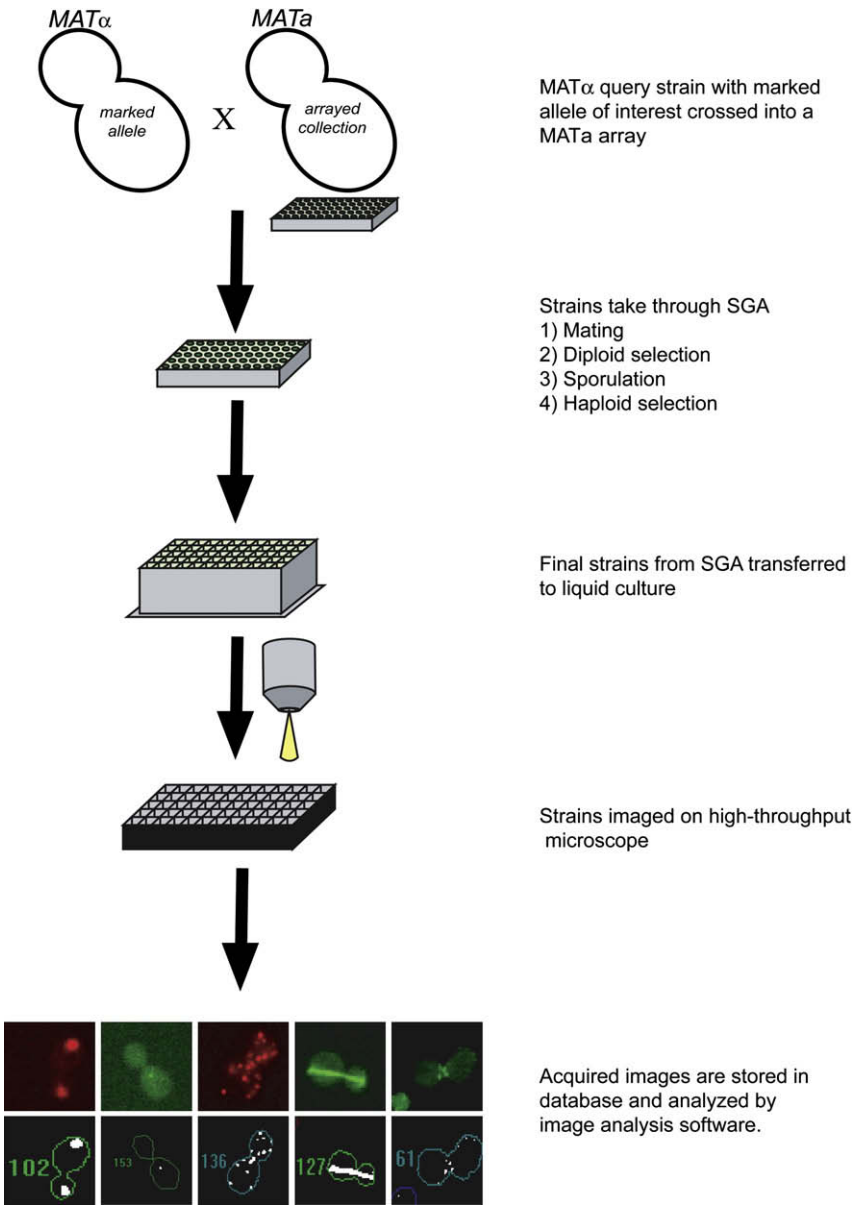

Fig. 1. Steps involved in high-content screening. Using the SGA methodology, a query strain expressing a fluorescent marker can be crossed with the arrayed deletion collection resulting in the expression of the fluorescent marker in the entire deletion array. These samples are then grown as arrays of liquid cultures and transferred to optical plates for imaging. The automated image analysis software, MetaXpress, can detect the specific signal of interest and measure its morphologic features.

is treated, which may be important for certain experiments. Since we are imaging live cells, we adopt a static protocol in which every plate is treated the same way leading to minimum variability between plates.

2.3. Automated image analysis and extracting key features

Once images are acquired, the next crucial step is to analyze the terabytes of data associated with a screen. Automation of this process not only yields high reproducibility, but also helps to identify subtle phenotypic differences in some cases [46]. Most automated systems come with a commercial software package that allows for sophisticated image analysis. Several software solutions, both open-source and commercial, are also available. For example, Definiens Software (http://www.definiens.com/) has been widely applied from Earth to Life science [47]. We have used MetaXpress to analyze our data as it includes several useful segmentation algorithms, and allows plugins to be built in C++. MetaXpress offers good versatility with an easy output of several morphometric features and unlimited calculation of positioning features. Academic laboratories have produced open-source algorithms that provide a cost-effective option and can be customized to individual assays. One such application that we have currently employed is CellProfiler [48]. CellProfiler is MATLab-based software, developed at the Broad Institute, with useful cell segmentation abilities (http://www.cellprofiler.org/). CalMorph is a software package developed specifically for yeast cell segmentation and morphometric and geometric analysis, and has been used for automated image analysis of fixed cells [33] and to investigate the natural genetic diversity of cellular morphology [49].

All automated image analysis software uses machine learning techniques that include the ability to automatically query images in a database, extract key morphometric features and export the measurements as a separate output. The image analysis process involves four basic steps: (1) pre-processing provides shade correction and background subtraction; (2) segmentation identifies all

cells or objects in each image; (3) classification places objects identified as 'regions of interest' into sub-populations, and (4) morphological measurement provides quantitative data on important features, enabling production of a unique morphometric profile for the cells being examined. For example, a minimal set of features can be used to train the imaging software to classify yeast cells based on their bud morphology which serves as a proxy for cell cycle position. This was useful in our recent study in which we used SGA to cross a GFP-Tub1 fusion protein into the yeast deletion collection in order to discover mutants that affect the dynamics of the mitotic spindle. We focussed on three spindle features – length, orientation and positioning – and discovered several new genes that regulate spindle disassembly [37]. By combining the spindle measurements with budding index, we were able to track the dynamics of spindle assembly and disassembly relative to cell cycle position in the entire deletion collection. For more complex phenotypes, such as aberrant morphology, the extraction of many features, including intensity values and texture measurements as well as morphometric features, is often required [17,27]. Non-redundant features can then be selected using dimensionality reduction [50].

As outlined above, automated image analysis of a single biological marker in a series of images is challenging. Additional computational challenges are faced when attempting to survey a series of mutant strains for a variety of patterns, such as the subcellular localization of different proteins. Recent progress has been made in this area, and suggests that the performance of automated image analyses in yeast cells, using a combination of both supervised and unsupervised learning, is more objective, quantitative and reproducible than manual inspection [51]. Developments in this area will be incredibly useful, as more laboratories apply automated image analysis to study a variety of cell biological phenotypes.

2.4. Data mining and hit identification

Data derived from HCS screens can be images, feature measurements or metadata. Storage and retrieval of data can be accomplished with a variety of database management tools; however, statistical analysis and data mining for multiplexed high content analysis (HCA) is still in its infancy. Challenges include: (1) the number of cells and features being assayed which creates large datasets that can overwhelm computational capacity when complex models are to be derived; (2) many conventional statistical analyses may not be applicable as some morphometric features do not follow a normal distribution; (3) phenotypic variability between plates and between experiments due to high volumes of sample processing may cause false positives; and (4) identification of key informative features from the many features measured to define patterns for functional prediction is highly dependent on the features chosen. Hence, most HCA are custom designed and also require manual inspection of images [14,15,22,27,28,52,53].

Recently, machine learning has been applied in segmentation of images to recognize specific patterns [51], and also to discover inherent properties of imaging data that differentiate mutant phenotypes from wild-type and allow functional predictions. For example, Ohya et al. used a support vector machine (SVM) to transform data to a high dimensional space through kernel mapping and to predict gene functions based on morphological profiles [33]. Similarly, Bakal et al. trained a neural network where a non-linear mapping of the original dataset was done by minimizing a certain objective function to identify local signaling networks that regulate cell protrusion, adhesion and tension [22]. For our analysis of spindle morphology in yeast mutants [37], we employed a machine learning technique that used a mixture of Gaussian models to learn the probability density function of control (wild-type) samples based on a subset of spindle features. Each mutant strain was then evaluated by computing the likelihood of significant variation from the control under this learned model by a random sampling procedure, using data from individual cells and generating a *p*-value. The significant deviation of the measurement values from those of wild-type cells reflect morphological defects and indicate a perturbation of the cellular process being surveyed. Accordingly, mutant strains with low *p*-values have spindle measurements significantly different from wild-type and are identified as defective in spindle dynamics [37]. Currently, there are no well-accepted standard procedures available for HCA and the continued development of standard statistical tools to process HCS data without sacrificing information accuracy will be important for the field.

3. Tools for HCS and their applications

3.1. Molecular tools

HCS approaches arguably revolutionized cell biology and its applications are expanding as more and more researchers adopt the technology. As noted above, a major advantage of yeast as a model system for high-content screening is the availability of many genome-wide collections of yeast strains for systematic analysis. Of particular note for high-content screening is the GFP-ORF collection in which about three quarters of the yeast genes are endogenously tagged with GFP at their C-terminus [35]. The collection is now being productively used to screen for both changes in localization and abundance of the yeast proteome. For example, Benanti et al. screened for substrates of the F-box protein Grr1p, which is involved in targeted proteolysis. By monitoring for changes in protein abundance, they were able to identify at least seven novel metabolic targets of Grr1p. The project involved introduction of an RFP marker linked to the *grr1Δ* locus into the yeast GFP collection using an SGA protocol. *GRR1* and *grr1Δ* cells were imaged simultaneously in the same well using a Cellomics high-throughput microscope system (40× objective). All the cells were identified using the stain CellTracker, which localizes to the yeast vacuole, whereas *grr1Δ* cells were distinguished from wild-type cells by the presence of the RFP signal. Once wild-type and mutant cells were identified, GFP intensity between null and wild-type cells was compared to assess changes in protein abundance.

A similar approach can be used to track genome-wide changes in protein localization caused by a genetic, chemical or environmental perturbation. In our laboratory, we are using SGA to introduce marked deletion alleles of interest, together with a constitutively expressed cytosolic marker, into the GFP-ORF collection. The cytosolic marker allows normalization of the varying GFP signals. We analyze images using Cell Profiler and features are extracted to assist in defining changes in proteins level or localization within the cell. This system allows us to globally track each tagged protein and the changes it undergoes at the cellular level. Integrating SGA analysis and HCS provides a general strategy for quantitative assessment of cell biological phenotypes on a genome-wide scale and in sensitized genetic backgrounds. A quantitative readout allows for the identification of subtle but significant phenotypes, offering the potential to confirm and expand genetic networks based solely on fitness measurements.

3.2. Reagents for HCS

Fluorescent labels and probes (Fluorescein and Rhodamine) that are covalently linked to substances that bind biomolecules or that bind biomolecules themselves (DAPI) are also useful for simple screens in HCS experiments. These types of reagents are particularly valuable when the addition of a GFP or other tag disrupts protein function [54]. In such situations, the small size of organic

fluorescent labels becomes useful. For example, the FlAsH (Fluorescein Arsenical Hairpin) reagent binds a small peptide tag (the CCXXCC motif) and can be used to fluorescently label recombinant proteins in live cells [55]. The high affinity of tetracysteine residues for organo-arsenical compounds promotes covalent bonds between the tetracysteine residues with the thioarsolan groups of the FlAsH reagent. Although this reagent has not been used yet in HCS, the background staining due to non-specific binding of FlAsH could be used to successfully segment whole cell information. Similarly a few other covalent labeling techniques such as SNAP-tag (New England Biolabs) and Halo tags (Promega) are potential tools for HCS. Methods have also evolved to monitor dynamic environmental changes within the cell. For example, a pH-sensitive GFP or an organic label could be employed to study membrane potential or ionic flux [56].

Several new approaches have been developed that combine clever genome-wide reagents with simple fluorescent readouts to map protein interactions [57]. Of specific interest, Michnick and colleagues have applied a fluorescent protein-fragment complementation assay (PCA) to detect spatio and temporal perturbations of protein–protein interactions in mammalian cells, following addition of drugs, siRNAs or hormones [58]. A specific application of a PCA assay called GePPI (genetic perturbations of sentinel protein interactions) was recently used to identify proteins involved in the regulation of a yeast cell cycle transcription factor [59]. The GePPI assay involves detecting interactions of fusion chimeras between proteins of interest that query a particular pathway and two fragments of the yellow fluorescent protein "Venus". When the Venus protein fragments are brought together due to a protein–protein interaction, a fluorescence signal is produced. Expression of these fusion chimeras in specific deletion backgrounds leads to loss of fluorescence signal revealing modulators of the pathway. With appropriate reagents, the GePPI and related assays could be combined with HCS platforms to provide genome-wide information about contextual interactions within signaling complexes and biological pathways.

3.3. Software tools

To quickly interrogate HCS data, commercially available packages that either come with the microscope or are sold separately are useful. These packages include: (1) Spotfire (http://spotfire.tibco.com/); (2) AcuityXpress (Molecular Devices); (3) CellmineT (http://www.bioimagene.com/); (4) Accelrys (http://accelrys.com/); (5) IDBS-Activity base suite (http://www.idbs.com/ActivityBase/); and (6) GeneData Screener (http://www.genedata.com/). These analysis packages allow quick assessment of data quality with a range of configurable components to create custom data processing workflows. In addition, several pathway-mapping tools are also being productively applied to the analysis and interpretation of HCS data. These tools have been typically used to describe biological networks in large-scale genomic datasets [60]. Examples include Pathway Studio (http://www.ariadnegenomics.com), Ingenuity Pathway Analysis (http://www.ingenuity.com/), Cytoscape (http://cytoscape.org/), Mapman (http://www.gabipd.org/), PathwayVoyager (http://www.genome.ad.jp/), MetaCore (http://www.genego.com/) and Pathart (http://www.jubilantbiosys.com/).

Open source platforms such as Open Microscopy Environment (http://www.openmicroscopy.org/) can also be used to visualize, manage and annotate images and metadata. In collaboration with a number of commercial entities, researchers at the University of Dundee developed a package that supports modeling workflows for the quantitative analysis of microscopy images. Academic labs have not only built image analysis software but also have developed programs that have the ability to control hardware. Micromanager (μManager), an open-source Java-based imaging software developed by the Vale laboratory (http://www.micromanager.org/), is a low-cost software platform for automated microscopy. When integrated with ImageJ software (http://rsb.info.nih.gov/ij), μManager implements several imaging procedures with the ability to design customized user plugins for specialized imaging applications.

Some useful databases have been developed for the storage and analysis of HCS data. As part of an effort to study lipid metabolism, Kohlwein and colleagues have started a relational database (http://ypl.uni-graz.at/pages/home.html) that stores images for yeast protein localization in order to accommodate the wealth of data emerging from large-scale genomic analyses. This open-access platform for submitting and storing image data allows the integration of specific information regarding experiments that are relevant to researchers [41]. In a complementary effort, Kumar and colleagues have built a cross-species organelle database (http://organelledb.lsi.umich.edu/) which houses information about the localization of proteins from 138 organisms with an emphasis on the major model systems. These open-access platforms allow integration of HCS and other functional genomic datasets and are an important community resource for understanding gene function.

4. Concluding remarks

Completion of the *S. cerevisiae* genome sequencing project catalyzed major efforts to create reagents and tools for functional annotation of genes. The combination of genomic approaches such as transcriptome profiling, organellar proteomics, database mining, comparative genomic analysis and standardized phenotypic analyses promises to produce a new global view of a model eukaryotic cell. Due to the logistics of imaging and tedious manual scoring of aberrant morphological phenotypes, large-scale cell biological studies have been a major challenge. However, significant progress has been made in the development of useful reagent sets and accessible microscope and software systems. While the current focus is on producing and analyzing still images, future methodologies may collect four-dimensional spatial and temporal information with multiple colors for different molecular reporters. The implementation of automated robotics for handling and imaging of cell cultures, allowing for rapid data acquisition, will vastly change the scope of genomic investigation in yeast.

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