



Using time-lapse fluorescence microscopy to study gene regulation

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ABSTRACT

Time-lapse fluorescence microscopy is a powerful tool to study gene regulation. By probing fluorescent signals in single cells over extended period of time, this method can be used to study the dynamics, noise, movement, memory, inheritance, and coordination, of gene expression during cell growth, development, and differentiation. In combination with a flow-cell device, it can also measure gene regulation by external stimuli. Due to the single cell nature and the spatial/temporal capacity, this method can often provide information that is hard to get using other methods. Here, we review the standard experimental procedures and new technical developments in this field.

1. Introduction

Time-lapse fluorescence microscopy is a powerful tool to study gene expression [1]. In this method, protein or mRNA tagged by fluorescence protein (FP) is imaged in real time in individual cells that are actively growing and dividing. Comparing with other biochemical methods in quantifying mRNAs or proteins, such as Northern/Western blots, reverse transcription polymerase chain reaction (RT-PCR), and RNA-seq, time-lapse fluorescence microscopy has a number of advantages. First, by measuring the fluorescence intensity of diffusive proteins or counting the number of tagged mRNAs, this method is highly quantitative. Based on our experience, the fluorescence measurement as a population average has smaller technical variability in comparison to other quantitative methods such as RT-PCR. Second, this method quantifies fluorescence in single cells. Therefore, it not only measures the protein/mRNA level as the ensemble average, but also generates information about the cell-to-cell variability (noise) of gene expression [2,3]. Third, this method yields spatial information. Instead of lysing the cells and completely disrupting their internal structure, fluorescent imaging detects the localization of the tagged protein/mRNA inside the cells. Fourth, unlike the “snap-shots” method such as flow cytometry where a cell is visualized only once, the time-lapse measurements keep tracks of individual cells over time. Such temporal capacity allows the measurement of gene expression dynamics; when cells are tracked over multiple generations, it also allows the evaluation of memory and inheritance, i.e. how the level of gene expression is maintained in a cell over time, or propagated from mother to daughter cells. Combining the temporal and spatial information, one can probe the movement of proteins/mRNAs inside cells; one can also study how gene expression is

coordinated among different cells, e.g. during embryonic development. Fifth, in addition to fluorescence, this method can simultaneously record other cellular conditions, including cell size, age, and cell cycle stage. By dividing cells in terms of their size or cell cycle stage and analyzing gene expression in these cells separately, this method circumvents the need of homogenizing or synchronizing the cells, therefore avoiding the artifacts generated in these processes. Finally, combining time-lapse fluorescence microscopy with flow-cell devices, one can investigate live cells in a variable micro-environment. For example, through media exchange, gene of interest (GOI) can be induced or repressed in a highly controlled manner, allowing us to probe how cells respond to environmental cues.

With all the power of time-lapse fluorescence microscopy, this technique also has its trade-offs. Protein/mRNA tagging can be challenging, especially in species with limited genetic tools; the throughput of the method is low because only a few types of proteins/mRNAs can be monitored at the same time; technical issues such as photo-damage, slow FP maturation rate, and low signal-to-noise ratio can significantly limit the application of the method and need to be carefully considered. Fortunately, progress is being made in all of these areas. Here, we review the standard experimental procedures and new technical developments of using time-lapse fluorescence microscopy to study gene expression dynamics (mostly at the protein level). Because of our expertise in budding yeast, many examples in the review are based on yeast, but the underlying principle should generally apply to other species.

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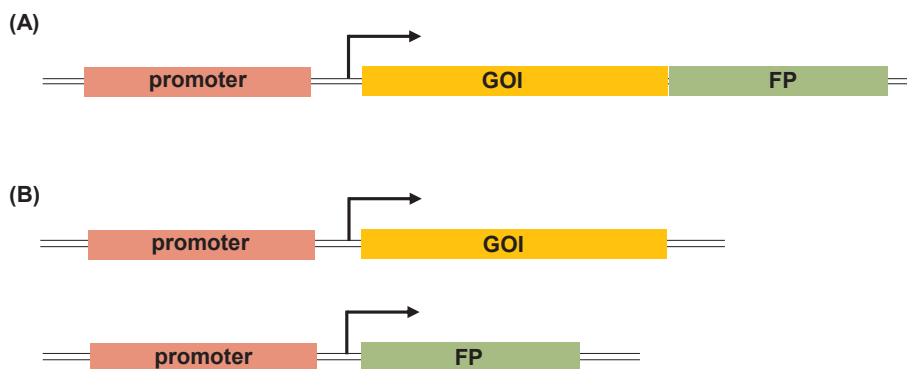


Fig. 1. Two ways to investigate gene expression. A) The endogenous gene of interest (GOI) is tagged by a FP. B) The endogenous GOI is intact, while the promoter driving FP is inserted into another location in the genome.

2. Experimental procedure

2.1. Use FP to probe gene activity

To use time-lapse fluorescence microscopy to study gene expression, the first step is to generate a fluorescent reporter for the GOI. One common strategy is to tag the GOI with a FP coding sequence, so that the fluorescence intensity and distribution reflect the concentration and localization of the fusion protein (Fig. 1A). One potential concern here is that the FP tag may change the function or the stability of the GOI; sometimes, different tagging configurations (N- or C-terminal, linker sequences, etc) need to be experimented to test the functionality [4,5]. Alternatively, the enhancer/promoter of the GOI can be used to drive the FP by itself, so that the FP intensity directly reflects the activity of these regulatory sequences (Fig. 1B). Such a construct does not perturb any endogenous genes, therefore alleviates the concern above. In addition, because the FP maturation and degradation rate will not be affected by any proteins attached to it, the activity between different enhancers and promoters driving the same FP can be directly compared. The drawback is that this construct cannot provide the information of protein localization. Although the constructs in Fig. 1 can be introduced into cells on a plasmid through transfection, the number of plasmids inside the nucleus is hard to control. For quantitative measurements, we usually integrate the FP reporter into the genome.

2.2. Considerations of FP label

Although a full spectrum of FPs is available for fluorescent labeling, they need to be carefully selected based on the detailed experimental setting. The most important parameters to consider here include the FP spectrum, brightness, stability, and the maturation/degradation rate.

2.2.1. Spectrum and brightness

Cells under continuous imaging are repeatedly exposed in excitation

light that may cause DNA damage, cell cycle arrest, and ultimately, cell death. In fact, photo-damage can often be the limiting factor in live-cell imaging. Cells from different species, or of different cell types, can have different sensitivity to light exposure [6], e.g. yeast is more resilient to strong excitation than mammalian cells. Across the spectrum, cells in general are more sensitive to excitations with shorter wavelength (like UV). Therefore, red-shifted FPs such as GFP, YFP, and mCherry are usually preferred over blue-shifted ones such as CFP.

Reducing photo-damage also means that the excitation should be minimized. At low excitation, weak and noisy fluorescent signal becomes a major issue for proteins of low abundance. To enhance the signal-to-noise ratio, one can increase the signal by using FPs with high quantum yield (brightness), and/or by choosing appropriate fluorescent channels with low background auto-fluorescence. For example, although GFP has higher quantum yield than mCherry, budding yeast grown in the synthetic complete media has much higher auto-fluorescence in the green than the red channel. Accordingly, we found that weak signals from mCherry can be better distinguished from the background than those from GFP.

2.2.2. Maturation and degradation rate of FPs

When choosing FP, its maturation/degradation kinetics is another important concern. The intensity of FPs is determined by its production, maturation, and degradation rate (Fig. 2A). In many cases, the latter two are intrinsic properties of the FP, and the production rate k_p is what we are interested in. When time-lapse fluorescence imaging is used to measure the changes in k_p , FPs with fast maturation and degradation are often desired as their intensities follow the k_p more dynamically (Fig. 2B). The maturation rate of FPs changes in different species. In yeast, EGFP and Venus are considered as fast maturing with half-life of 15 and 18 mins [7]. In contrast, another commonly used FP, mCherry, has a much slower maturation rate of 45 min [8]. These proteins by themselves are stable, i.e. when the production of these proteins is shut off, their concentration will be gradually diluted due

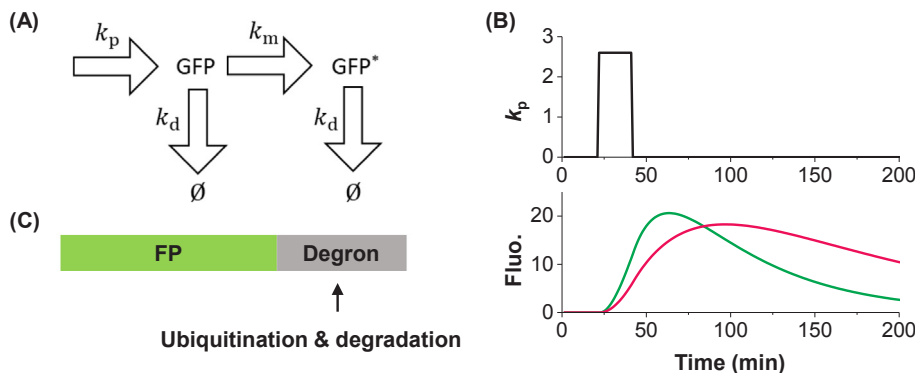


Fig. 2. Maturation and degradation processes of FPs. A) FP production and maturation process. The production, maturation, and degradation rates of the FP are labeled as k_p , k_m , and k_d . B) Simulation of fluorescent dynamics of two FPs with different maturation and degradation rates. The activation occurs as a pulse during a short window of time. The two curves below are the fluorescence readout from FP with fast maturation and degradation rate (green) or with slow ones (red). C) Fusing FP with sequence targeted by ubiquitination can increase its degradation rate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to cell growth, and the fluorescence intensity will follow an exponentially decay with a time constant close to the cell doubling time. For some applications, degradation rate can be increased by attaching a degron sequence to the FP, so that the protein will be ubiquitinated and actively degraded by proteasome (Fig. 2C) [9]. In yeast, fusing Venus with destruction sequence in *CLN2* reduces the half-life of the protein from ~100 min to ~39 min [7]. Such destabilized protein is often used to study rapid changes in promoter activities for cell-cycle regulated genes [10–12]. Similar strategies can be employed in mammalian cells to reduce the half-life of GFP from 26 hrs to a few hrs [13].

It should be noted that high degradation rate comes at the cost of FP intensity. In an extreme case, if all the FPs are degraded before they reach maturation, there would be no fluorescence signal. This can be a problem when FPs are used to label some proteins with high turnover rates. A recent study solved this problem by pre-expressing FPs while engineering a tag on the GOI that binds to the matured FP [14]. This method circumvents the limit imposed by the maturation rate, and was used to visualize some very unstable transcription factors in live *Drosophila* embryos.

2.2.3. Differentiating weak signal from auto-fluorescence with localization

When a fluorescence signal becomes so weak that it is comparable to that of auto-fluorescence (which changes from cell to cell and shows cell-cycle dependent changes), we used a localization trick to separate them [15]. Fig. 3A & B illustrates a case where the auto-fluorescence of a yeast cell shows the same dynamic changes over the cell cycle as a FP-labeled cell-cycle regulated protein. By adding a nucleus localization sequence (NLS) to the FP, the real signal concentrates in the nucleus, which can be distinguished from the diffusive background (Fig. 3C & D). Besides NLS, other localization signals may also serve the same purpose.

2.2.4. Multiplexing of FPs

Imaging more than one factor in the same cell necessitates multiplexing of FPs. FPs all have broad, overlapping spectra, which limit the number of FPs that can be used simultaneously. More FPs also means higher exposure and more photo-damage to the cells. For time-lapse live-cell imaging, the latter imposes the real limit: up to 10 fluorophores can be used for immunostaining in fixed cells, while FPs used in time-lapse measurements rarely exceed 3–4 types. When choosing two FPs, if the maturation kinetics is not a concern (e.g. only the steady-state levels of FPs are measured), one should pick FPs with minimal overlapping spectra. EGFP and mCherry can be a good combination. When the FPs

are dynamically regulated, and their intensities need to be directly compared (e.g. use the same promoter driving different FPs in the same cell to quantify intrinsic vs extrinsic noise), the pair of EGFP and Venus can be used. However, the two signals need to be mathematically decoupled because the two FPs have significantly overlapping spectra [16]. Such treatment inevitably decreases the signal-to-noise ratio of the measurement.

2.3. Sample preparation

In this section, we describe the preparation of live cells for time-lapse imaging. This step varies significantly among different species, and can also be different due to microscope setting. Here, we focus on making yeast samples using epifluorescence microscopy.

2.3.1. Agarose pad sample

Epifluorescence microscopy requires the sample to form a flat 2D layer. Piling up of the cells in 3D seriously compromises the image quality. When there is no need to change media during the time-lapse imaging, an agarose pad can be used to confine the cells (Fig. 4A) [10,17]. Culture of yeast cells is first grown in synthetic media to log-phase with optical density (OD) ~ 0.1, and sonicated lightly into single cells. The choice of media here is very important: rich YPD media cannot be used because it generates very high auto-fluorescence; regular synthetic complete media are suitable for most applications; for some very weak signals, low-fluorescence media without riboflavin and folic acid would help to enhance the signal-to-noise ratio [18]. Low melting agarose is then dissolved in this synthetic media with w/w ratio 1.5%. After the agarose solution solidifies into a thin pad between two slides and cut into small squares, a small amount of yeast culture (~1 μ L) is dropped onto each pad and covered with a coverslip (Fig. 4A). Yeast will continue to grow in the 2D plane between the agarose pad and the coverslip with doubling time similar to that in liquid media. If the sample needs to be pre-incubated before mounting onto the microscope, it should be kept in a moisture environment to prevent drying. These cells typically can be imaged for 8–10 hrs before they become too crowded and start to pile up on each other.

2.3.2. Flow-cell sample

Flow-cell allows us to image cells while changing their environment. There are different types of flow-cells, but they all have 1) chambers where cells can grow, 2) channels allowing the exchange of media, and 3) a mechanism to immobilize the cells despite the media

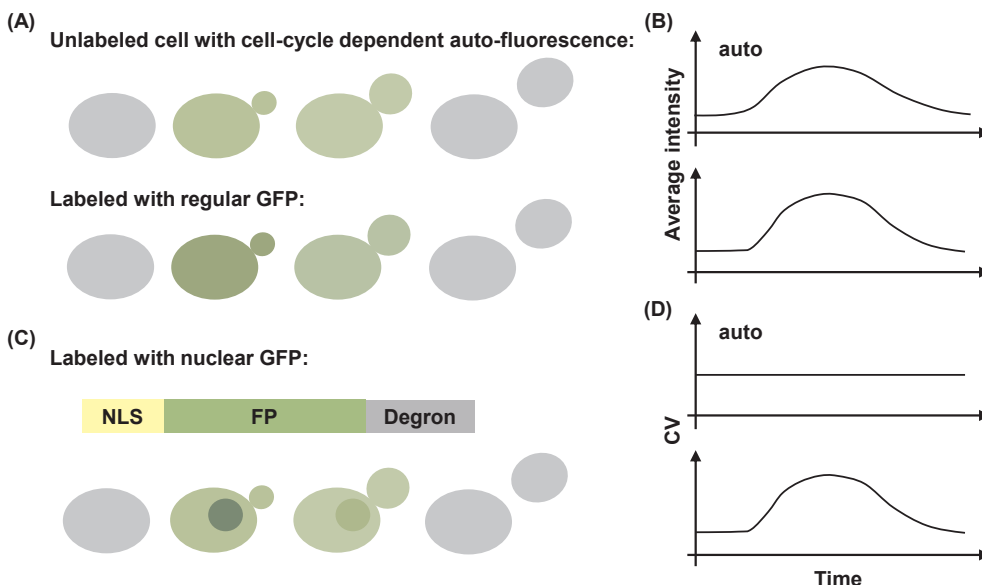


Fig. 3. Differentiating weak signal from autofluorescence. A & B) Cell-cycle dependent auto-fluorescence signal in unlabeled yeast can be hard to differentiate from real cell-cycle regulated FP signal, especially when they show similar pattern of oscillation. C & D) Using nuclear FP to differentiate the real signal from auto-fluorescence. Unlike the auto-fluorescence, which is uniform inside the cells (low variance throughout the cell-cycle), the real signal accumulates inside the nucleus, causing high CV.

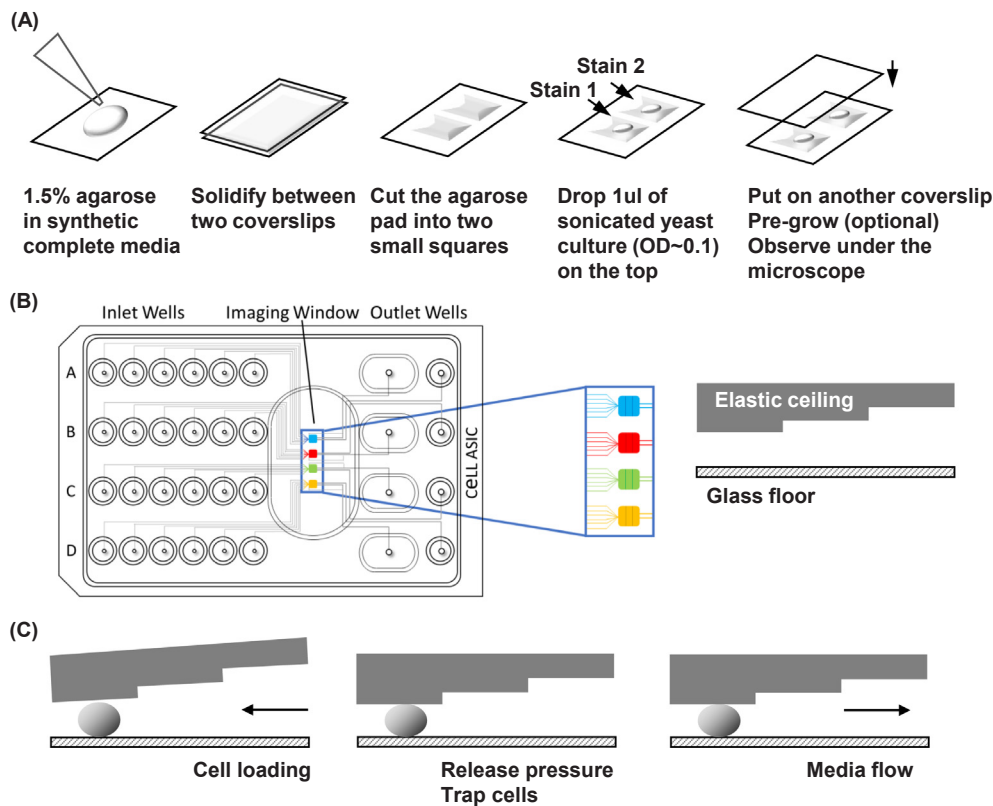


Fig. 4. Sample preparation. A) The agarose pad method. B) The flow cell device from CellASIC®. C) Using the flow cell to load cells, hold cells, and change media.

flow. Our lab routinely uses CELLASIC® ONIX microfluidic plate, which contains cell chambers confined by an elastic ceiling and a glass floor (Fig. 4B). The elastic ceiling consists of three steps that can capture cells with various size. The plate is connected to a control system that generates pressure and forces the liquid flow through the inlet and outlet wells. While loading the cells, the elastic ceiling is distorted under the pressure, allowing the cells to enter the chamber (Fig. 4C). During media perfusion, the elastic ceiling restores to its original position and clamps down on the cells. Using a commercial software, one can design the sequence, flow rate, and duration of the media flow, which runs automatically during the image acquisition. Besides the capability of media exchange, flow-cell also provides a stronger confinement of the yeast cells inside a 2D space, allowing them to be imaged for longer time (up to 15 hrs).

2.4. Microscope setting and image acquisition

2.4.1. Considerations in the microscope configuration

Besides standard measures to achieve high resolution imaging (e.g. high NA objective, high-performance camera, etc.), the more specific challenge for long-term imaging is to efficiently detect the fluorescence signals with highest possible signal-to-noise ratio while maintaining low exposure to excitation light to avoid photo-damage to the cells. We have discussed in a previous section that certain choice of FPs can help reduce the photo-damage. A few microscope settings are also critical for this purpose.

- 1) The light source should have narrower bandwidth close to the absorption maximum [6]. Although FPs tend to have wide spectrum of excitation/emission, they have different absorption efficiency at different wavelength. The excitation light with a wavelength different from the absorption maxima contributes less to the fluorescence signal, but can cause significant photo-damage to the cells, especially at shorter wavelengths. Indeed, excitation passed through

narrow-band filters was shown to cause less cell damage than broad-band excitation with the same total intensity [6].

- 2) Excitation and image acquisition should be synchronized. In our microscope (Leica DMI6000 B), there is an internal shutter controlling the passage of the excitation beam, but it tends to be out of register with the image acquisition, causing wasteful exposure as well as inaccuracy in the measurement. This problem can be solved by an external shutter, which directly triggers the camera through a voltage signal, and therefore synchronizes precisely with the camera.
- 3) Using light sheet microscopy to reduce exposure. Imaging sub-cellular object often requires taking z-stacks along the axial direction to find the focus plane. During z-stack imaging, the cells are repeatedly exposed to the excitation light, dramatically increasing the photo-toxicity. In contrast, in light sheet microscopy, the excitation light is expanded in one dimension by a cylinder lens to provide a thin layer of illumination (Fig. 5) [19,20]. During z-scanning, the cells are illuminated layer-by-layer sequentially without repeated exposure. In addition, this design enhances the signal-to-noise ratio of the image by eliminating the contamination of the fluorescence signal generated by the off-focal planes.
- 4) Another important setting is the excitation intensity and exposure time, where the latter imposes a constraint on the time resolution (frame-rate) of the data acquisition. The frame-rate is ultimately limited by the hardware: point-scanning confocal microscope, for example, typically takes one frame per second, while epifluorescence, line-scanning or spinning-disk confocal microscopes can image much faster. For applications where high temporal 3D imaging is required (e.g. tracking the movement of a single mRNA molecule in live cells), multifocus microscopy can further increase the image acquisition rate by simultaneously imaging different focal planes within the specimen [21]. Fortunately, in most gene regulation studies, the accumulation and degradation of the reporter FPs often occur on the time-scale of minutes, much slower than the

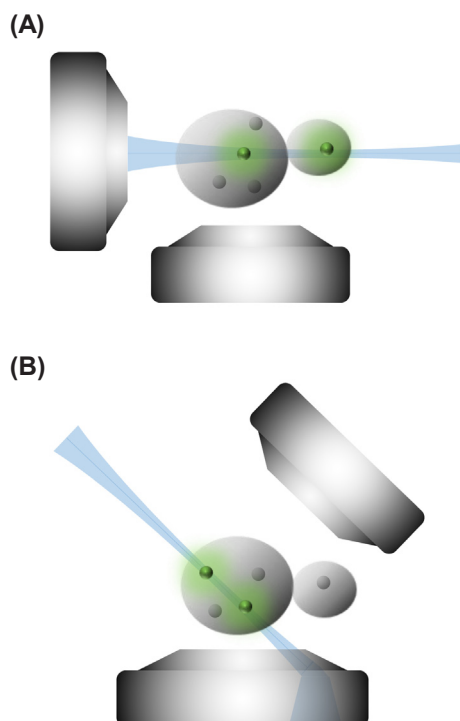


Fig. 5. Two types of light sheet microscopy. A) Single plane illumination microscopy [20]. The illumination objective on the left generates a thin layer of laser light onto the sample. The detection objective is placed underneath the sample. B) In the oblique single plane illumination microscopy, an off-axis incident beam passes through the edge of the objective and forms a tilted light sheet. The fluorescence is observed in the direction perpendicular to the light sheet [19].

limit mentioned above. For slowly-changing signals, one often wants to lower the excitation intensity and have extended data acquisition period: the combination of shorter exposure/higher excitation and longer exposure/lower excitation generate similar images, but the latter reduces photo-toxicity to the cells [6].

2.4.2. Automated image acquisition

Because time-lapse imaging can last hours, even days, it is crucial for the image acquisition process to be automated, i.e. the microscope should automatically go to the region of interest, focus, take images in different channels, and restart the process at the pre-set time points. There are many commercial or in-house software that enable such automation. Here, we describe the strategies used for the autofocus step, which is critical for time-lapse imaging due to constant mechanical or thermal drift of the focal point.

Some microscopes carry out autofocus through an optical module where an off-axis LED beam is reflected by the water/glass interface in the sample onto an optical detector (Fig. 6A). When the sample deviates from the pre-determined focal plane, the reflected LED beam will shift on the sensor, triggering a feedback to move it to the focal position. Such feedback can either be done by physically moving the objective using a piezoelectric stage, or with an electrically tunable lens that can adjust the focal point with a current [22]. These autofocus modules are accurate and fast, and since they are controlled internally by the microscope, they do not require any additional software. However, for this method to work, it is critical that the cells of interest all have the same distance to the surface that reflects the LED beam. If the cells are directly deposited on glass (like in Fig. 6A), this is usually not a problem. However, when the glass is coated, and the coating has uneven thickness, this autofocus system may not work well. In the latter case, software-based autofocus can be used.

The software method, although slower and less robust in

comparison with the optical method, does not require specialized hardware and is compatible with various microscopes and samples. By taking a series of phase-contrast images at different axial positions and calculating the “focus index” of each image based on its contrast and sharpness, one can fit the focus index curve with a Gaussian function where the peak indicates the optimal focus point (Fig. 6B) [23]. In our hands, this autofocus method occasionally fails, sometimes because the focus drifts out of range, or because some surface impurities hijack the software so that it tries to focus on the wrong object. More reliable algorithms will benefit future measurements.

2.5. Image analysis

One time-lapse measurement can generate hundreds of images, each containing multiple cells, making the data analysis extremely tedious. An automated image analysis program is needed for this process. Unfortunately, the way to identify cells, as well as the subsequent quantitative analyses, differs significantly among experiments, and the image analysis program often needs to be custom-made. Here, we list some steps that are typically included in the analyses.

- 1) Segmentation of the cells. The segmentation method strongly depends on the imaging techniques. Identification of the cells from the phase contrast image, which relies on the high contrast along cell boundaries, is very different from doing so with a diffusive fluorescence signal that fills the whole cellular area. Again, because fluorescence imaging introduces more photo-damage, phase contrast is more commonly used for this purpose. For our analysis, we first separate the yeast clusters from the background based on the borders, and within each cluster, identify the contours for individual cells using the intensity gradient at the edge (Fig. 7A).
- 2) Tracking cells over time. Cells grow in size, change shape, and move during active migration or colony growth (Fig. 7B). Time-lapse measurements usually choose time intervals so that the locations and morphologies of the cells remain similar between adjacent frames, making it possible to link the cells from one frame to another. Two cells sharing high similarity in position, shape, and size can be annotated as the same cell; repeating this mapping process over all the frames allows us to trace the cells over long time. Tracking cells in tissues or during embryonic development can be more difficult due to the crowded environment and/or fast movement of cells. One strategy is to “barcode” the cells through stable transgenes that express a random combination of multiple FPs [24]. For example, three FPs, each can be “on” or “off”, can label eight types of cells. Such information can help differentiate individual cells and trace them from frame to frame.
- 3) With the contour information of individual cells over time, their fluorescence signals can be extracted from each frame. For FP that diffuses throughout the cell, one can get the total fluorescence (sum of the FP intensity at all pixels within each cell boundary) or the average (total fluorescence normalized by the cell area). We usually use the latter since it reflects the concentration of the FP (Fig. 7C). For FP that accumulates in part of the cell, e.g. inside the nucleus, one can either get the nuclear boundary (e.g. using FP labeled histone genes) and directly calculate average intensity inside the nucleus, or use the variation of the pixel intensity inside the whole cell to estimate the nuclear FP level (more FPs in the nucleus leads to higher variation among pixels, like in Fig. 3C & D). Comparison of the fluorescence intensity among different cells provides information on gene expression noise; plotting the intensity as a function of time reveals gene expression dynamics.
- 4) Phylogenetic history of individual cells is often needed to understand how gene expression is propagated across generation. In crowded colony of budding yeast, it can be difficult to identify mother/daughter pairs, as a small bud can be adjacent to many cells. One trick is to use FP tagged myosin [25], which forms a ring around the

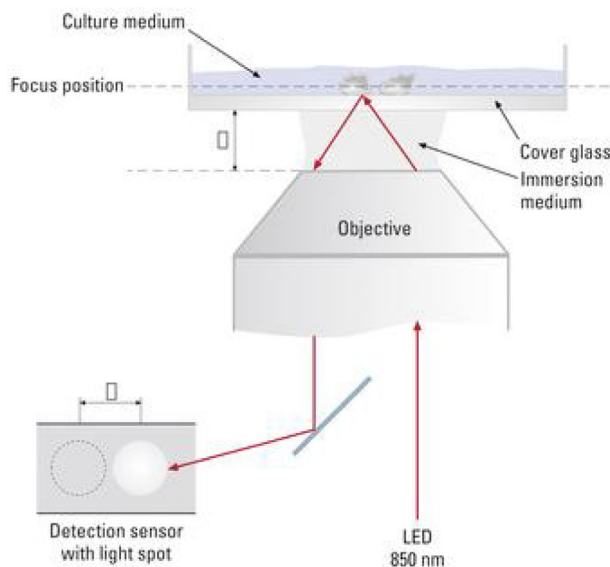
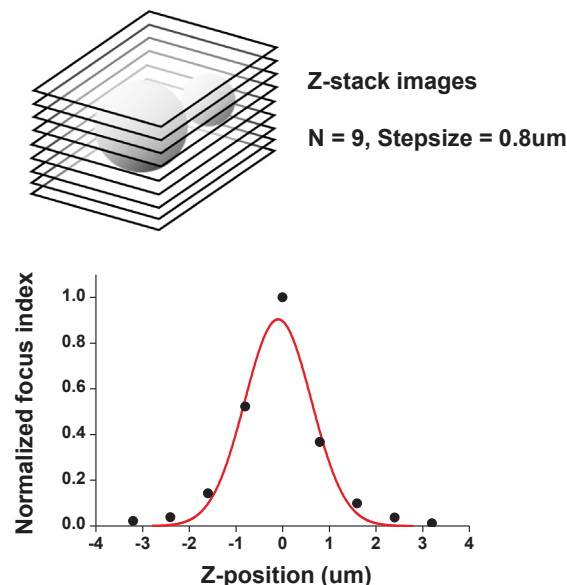
(A) Hardware autofocus:**(B) Software autofocus:**

Fig. 6. Autofocus method. A) Hardware autofocus (from Leica® Adaptive Focus Control; permission from Leica® to use the figure). B) Software autofocus by taking z-stack images. The optimal focal plane is determined by the “focus index” (lower panel), which is calculated based on the intensity and sharpness of the z-stack images.

budneck, establishing the mother-daughter relationship. In addition, the myosin signal also tells us when a cell is born, because it disappears rapidly upon cell division (Fig. 7D). Using these information, a phylogenetic tree of the colony can be constructed (Fig. 7E), and the expression level in mother/daughter cells can be compared. Inheritable expression of the “barcode” transgenes can also be used to trace the progenies of individual cells [26].

3. Summary and perspective

Time-lapse fluorescence microscopy has made major contributions to the study of gene regulation, and will continue to be one of the major tools in this field. Being a largely non-invasive method, it allows us to probe the concentration and localization of FPs or FP-labeled proteins in single cells in real time. Researchers have been using this method to study the dynamics [27–33], noise [12,16,34–38], movement [39,40], memory [11,41], inheritance [42–44], and coordination [45–48], of gene expression during cell growth, development and differentiation, sometimes in the presence of external perturbation. Due to the single cell nature and the spatial/temporal capacity, this method can often provide information that is hard to get using other methods.

There are a few factors that limit the application of time-lapse fluorescence microscopy. Photo-damage imposes a fundamental limit on how many photons can pass through cells before generating stress-response. To increase the observation time, one often need to sacrifice signal-to-noise ratio and spatial/temporal resolution. This problem can be tackled from two aspects. First is to develop better fluorophores to allow the detection of weaker signals with lower exposure. In recent years, organic fluorescent ligand has been used to label proteins in live cells through certain enzymes such as Halo and SNAP tags [49]. These enzymes are genetically fused to the GOI, expressed in the cells, and then catalyze a reaction that covalently conjugate a fluorescent dye to themselves. In comparison to FPs, these brighter and more stable dyes can even be visualized at the single molecule level inside the cells [50,51]. However, since unconjugated free dye needs to be washed out before imaging to ensure a low fluorescent background, only the proteins synthesized prior to washing can be visualized. This feature is not compatible with many gene regulation studies, where the newly synthesized proteins need to be continuously monitored. We expect future

developments in this field to solve this technical problem. The second aspect is the development of microscopy. As mentioned above, new imaging methods like the light-sheet microscopy can help reduce the phototoxicity. However, implementation of such microscope is still technically challenging [20], and this approach has not been widely used. In the next few years, we anticipate significant progress in terms of optical design, resolution, imaging speed, and image acquisition software.

Another limiting factor is the throughput – it is hard to simultaneously image many GOIs in large number of genetic backgrounds under different external conditions. This problem is partly solved by multi-well microfluidic chips that enable parallel processing of many different cells under different conditions [52–54]. Although the details of the design can be different, the general idea is to micro-fabricate PDMS devices containing hundreds or even thousands of cell chambers using soft lithography. Physical barriers and traps can be engineered into the chambers to efficiently capture cells. Time-lapse measurements are performed on these cells as they go through clonal expansion in these chambers with media perfusion. Many experiments can be done in parallel on these chips because each chamber can harbor a different kind of cells and/or under different conditions. Because of the high-throughput capacity, these devices are sometimes used to conduct genetic screening for certain growth phenotypes [55]. By analyzing a large number of cells, this method also allows the detection of rare phenotypes [56]. Microfluidic systems are also used for long-term imaging. With agarose pads or regular flow-cells, the sample is quickly saturated with exponentially growing cells, preventing us from probing long-term processes such as aging. In contrast, some microfluidic devices can remove newly-born cells by media flow so that the aged mother cells can be selectively image [57–59]. In the future, we expect to see more microfluidic devices with ease of use and more robust control.

Acknowledgements

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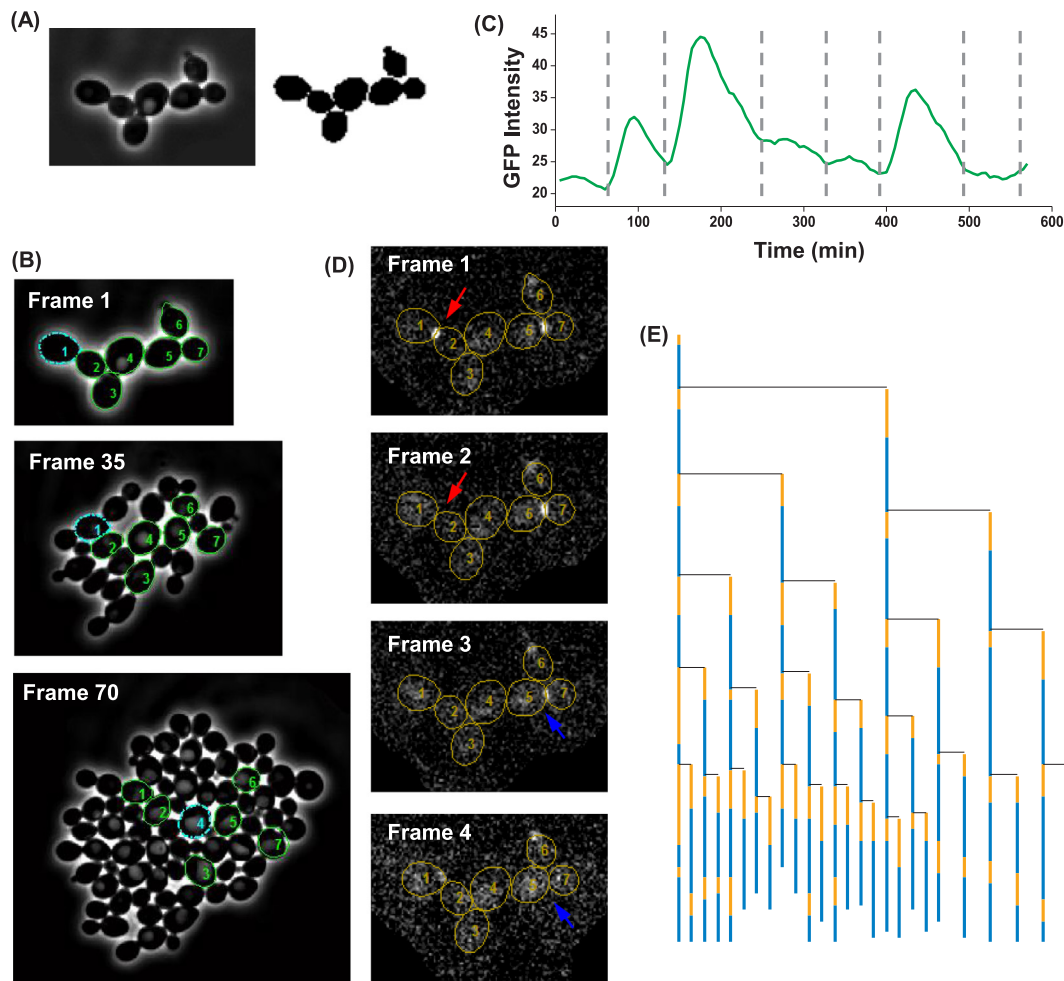


Fig. 7. Auto-segmentation and tracking cells in time-lapse fluorescence microscopy. A) Cell segmentation. The phase contrast image (left) was converted into a binary image (right), where cell contour information can be extracted. B) Tracking cells along different image frames. The same cell is identified by its morphology properties and annotated by the same number along the movie. C) One example of GFP intensity trace. The curve shows the green fluorescent intensity as a function of time in a single cell that contains the *HO* promoter driving GFP. Cell division times are marked as dashed lines. In this cell, *HO* promoter shows cell-cycle regulated activity in a fraction of cell cycles. D) The Myo1-mCherry fusion protein identifies mother/daughter pairs, and also provides the timing of cell division. E) The phylogenetic tree of one yeast micro-colony. The branching point represents the events of cell division.

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