1. YEAST METHODS ........................................................................................................... 2
  1.1 General yeast and nucleic acid manipulations ............................................................ 2
  1.2 Yeast strains .................................................................................................................. 3
  1.3 Plasmids ....................................................................................................................... 4
  1.4 Western blot quantification .......................................................................................... 5
  1.5 Quantification of numbers of Ste5 molecules in living cells with fluorescence .......... 6

2 IMAGE ANALYSIS AND DATA PROCESSING ......................................................... 6
  2.1 Automatic focus on the imaged cells ............................................................................ 6
  2.2 Identifying Single Cells in Bright Field Images with Cell-ID ....................................... 7
  2.3 Analysis of the statistics with PAW ........................................................................... 11
  2.4 Total fluorescence with Cell-ID ................................................................................ 12
  2.5 Using time course data to correct total fluorescence .................................................. 14
  2.6 Photobleaching correction to total fluorescence ....................................................... 14
  2.7 Point spread function and correction for out of focus light from the cell ..................... 15
  2.8 Correction for nonuniform intracellular distribution of fluorescence ......................... 20
  2.9 Examples of individual cell statistics ......................................................................... 21
  2.10 Volume calculation with Cell-ID ............................................................................. 23

3 RATE CALCULATIONS ................................................................................................. 26
  3.1 Measurement of fluorophore maturation rates ............................................................ 26
  3.2 Stability of YFP-ADH1tail and GFP-STE5 from galactose shut-off experiments .......... 29
  3.3 Snapshot experiments: calculation of degradation rates in vivo ............................... 30
1. Yeast methods
1.1 General yeast and nucleic acid manipulations

We performed nucleic acid and yeast manipulations as described \(^1,2\). We made and targeted PCR products for genomic integration as described \(^3,4\). To prepare cells for experiments, we grew strains in log phase for more than 15 hrs in defined synthetic medium lacking specific nutritional supplements as necessary to select the auxotrophic marker(s) and ensure the continued presence of recombinant constructs (appropriate Dropout medium \(^1\), BSM Bio-101 formulations, Qbiogene, Irvine, CA) with yeast nitrogen base (Difco, Becton Dickinson) and 2% dextrose, unless another carbon source is indicated.

Usually we performed our measurements using live cells. However, in the experiments shown in Figures S1c, 3 and 4d we fixed the cells before the measurement. To do that, we mixed the sample with an equal volume of 2\% paraformadehyde (pH=7.0) and incubated the cells for 1 hour on ice. Then, we washed the cells twice with PBS.

For the purpose of comparing the microscopic method with flow cytometry (Figure 3) we stimulated ACLY387 cells with different pheromone concentrations for 90 minutes in a test tube at 30\(^\circ\)C with shaking. To prevent pheromone binding non-specifically to plastic and glass, we diluted it into medium supplemented with 20 \(\mu\)g/ml of casein as described \(^5\). We then, fixed the cells and resuspended them in TE (pH=7.5), sonicated and then split into two tubes, one for microscopy and the other for flow cytometry. We used a Becton-Dikinson LSRII cytometer at its low flow rate setting. We excited YFP with a 25 mW 488 laser set at 500V. To detect fluorescence, we used the FITC channel (dichroic mirror high pass above 505 nm, em=515 to 545 nm).

For microscope observations, we observed cells with an inverted microscope through the glass bottom of 96-well plates (96 MicroWell Optical Bottom Plate #1.5 Coverglass Base, catalog no. 164588, Nalge Nunc International, Rochester, NY). We sonicated the cells to break apart clumps and added 100 \(\mu\)l of cell suspension (typically \(~10^6\) cells) to 96-well glass bottom plates that had been pre-coated with the lectin concanavalin A (conA, type V, Sigma-Aldrich) to provide a sticky surface to which the carbohydrate-rich yeast cell wall can attach. Using this procedure, cells remained attached for several hours, even after extensive washing and numerous horizontal (XY) translations of the microscope stage. In order to image multiple fields of cells automatically and repeatedly over time, we controlled the microscope and camera with commercially available software, MetaMorph (Molecular Devices, Downingtown, PA). We manually selected at least 3 representative fields of 50-100 cells in each well and recorded the stage positions, enabling the computer-controlled motorized stage to return repeatedly to the same fields and to capture their images at various times. The use of 96 well plates enabled us to expose cells to different conditions in parallel in each run. We coated the bottom of the plate with oil, so that a cushion of oil separated the lens from the
plate throughout the XY translations. We estimated that on average, 2.5 photons falling on any pixel of the CCD produced a single count in that pixel.

In typical time courses, we captured images every 15 minutes. For each field of cells at each time point, we usually acquired a single bright field image, then a YFP image, then, as relevant, a CFP image (YFP filter set 41028 ex 490-510 nm, em 515-550 nm and CFP filter set 31044v2 ex 425-445 nm, em 465-500 nm, Chroma Technologies Corp., Brattleboro, Vermont). We exposed YFP images for 1 sec and CFP for 0.1 sec.

We measured the discrimination between pheromone treated and untreated \( P_{\text{PRM1}} \) YFP cells by determining a threshold level of fluorescence that was such that 95% of the untreated cells lay below it. We then determined the fraction of cells stimulated with different levels of pheromone that had fluorescence above this threshold. The value of this fraction is the probability that a single cell drawn at random from a population treated with that amount of \( \alpha \) factor would be bright enough for us to assert with 95% or better confidence that it was not drawn from the untreated population.

### 1.2 Yeast strains

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Relevant Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACLY379</td>
<td>MATa bar1Δ</td>
</tr>
<tr>
<td>ACLY387</td>
<td>MATa bar1Δ Δprm1::( P_{\text{PRM1}} )-YFP::his5(^+)</td>
</tr>
<tr>
<td>TCY3096</td>
<td>MATa bar1Δ cdc28-as2 Δprm1::( P_{\text{PRM1}} )-YFP::his5(^+) trp1::( P_{\text{PRM1}} )-CFP-TRP1</td>
</tr>
<tr>
<td>TCY3154</td>
<td>MATa bar1Δ cdc28-as2 Δprm1::( P_{\text{PRM1}} )-YFP-his5(^+) trp1::( P_{\text{ACT1}} )-CFP::TRP1</td>
</tr>
<tr>
<td>TCY3056</td>
<td>MATa bar1Δ ste5::YFP-STE5</td>
</tr>
<tr>
<td>TCY3057</td>
<td>MATa bar1Δ ste5::YFP-STE5 trp1::( P_{\text{STE5}} )-YFP-STE5-TRP1</td>
</tr>
<tr>
<td>TCY3216</td>
<td>MATa bar1Δ ste5::YFP-STE5 trp1::( P_{\text{STE5}} )-YFP-STE5-TRP1 ura3:: 2X(( P_{\text{STE5}} )-YFP-STE5-URA3)</td>
</tr>
<tr>
<td>TCY3090</td>
<td>MATa bar1Δ ura3::( P_{\text{STE5}} )-YFP-URA3</td>
</tr>
<tr>
<td>TCY3039</td>
<td>MATa bar1Δ ste5::TRP1::( P_{\text{GAL1}} )-GFP-STE5</td>
</tr>
<tr>
<td>TCY3281</td>
<td>MATa bar1Δ ura3::( P_{\text{ACT1}} )-YFP</td>
</tr>
<tr>
<td>TCY3282</td>
<td>MATa bar1Δ ura3::( P_{\text{ACT1}} )-YFP-ADH1tail</td>
</tr>
<tr>
<td>TCY3255</td>
<td>MATa bar1Δ bar1::TRP1::( P_{\text{GAL1}} )-GFP</td>
</tr>
<tr>
<td>TCY3287</td>
<td>MATa bar1Δ ura3::pTC7-YFP-Adh1-tail::URA3</td>
</tr>
</tbody>
</table>

Strains used during this work. All were derived from W303a as described in the Supplementary Materials.

Strains are detailed in Table S1. The parent strain was YAS245-5C (can1::HO-CAN1 ho::HO-ADE2 ura3 ade2 leu2 trp1 his3) and is a W303a derivative \(^6\). W303a differs most significantly from S288C in that it is wild type for the Fus3-like protein kinase, Kss1. Construction of ACLY379, ACLY387, TCY3096 and TCY3154 is detailed elsewhere \(^5\). We constructed TCY3056 in two steps. First we integrated plasmid \( P_{\text{STE5}} \)-YFP-STE5-URA3 in the genome at the STE5 promoter (\( P_{\text{STE5}} \)). To do so, we linearized the plasmid with PshAI, transformed ACLY379 cells and selected colonies on uracil deficient plates. This resulted in an intermediate strain with two copies of the STE5
gene in tandem, one of which was a YFP-STE5 fusion. We then selected for strains in which URA3 and one of the STE5 genes had been looped out by homologous recombination, by plating the cells on 5-FOA (5-fluoroorotic acid, Sigma-Aldrich Inc, St. Louis, MO) plates. We screened by fluorescence microscopy for colonies that retained the YFP-STE5 fusion instead of the original STE5 gene, and confirmed expression of the YFP-Ste5 protein by western blot. We constructed TCY3057 by integrating p_{P_{STE5}-YFP-STE5-TRP1} into the trp1 locus of TCY3056, TCY3216 by integrating p_{P_{STE5}-YFP-STE5-URA3} into the ura3 locus of TCY3057 and TCY3090 by integrating p_{P_{STE5}-YFP-URA3} into the ura3 locus of ACY379. In all integrations, we selected for integration using the auxotrophic marker, screened recombination by PCR, and confirmed fusion protein production by fluorescence microscopy and Western blotting. We constructed TCY3255 by integrating a PCR product containing the TRP1 gene and P_{GAL1}-GFP followed by the ADH1 terminator, amplified from pFA6-TRP1-PGAL1-GFP, into the bar1 locus (replacing the region from nucleotides –200 upstream of the first base of the coding sequence and +20 from the stop codon) of ACY379. We constructed TCY3039 by integrating into ACY379 a PCR product containing the TRP1 gene upstream of a P_{GAL1}-GFP coding sequence, missing the stop codon, amplified from pFA6-TRP1-PGAL1-GFP, into the STE5 locus (at position +3 after the STE5 ATG), so that the GFP was fused in frame with STE5 starting at amino acid 2 of the STE5 coding sequence. Finally, we constructed TCY3287, TCY3281 and TCY3282 by integrating at the ura3 locus of ACY379 plasmids p_{P_{GAL1}-YFP-A206K-ADH1tail}, p_{P_{ACT1}-YFP-A206K} and P_{ACT1}-YFP-A206K-ADH1tail.

Cells expressing YFP-Ste5 instead of native Ste5 have a functional pheromone pathway, as determined by their ability to arrest cell cycle progression, initiate morphological changes, induce pheromone response genes, and mate normally (not shown). After transformation of a strain with YFP or YFP-Ste5 containing plasmids, we grew cells from single transformant colonies and measured their YFP signal as in this paper. We observed that each colony displayed a cell-to-cell distribution of YFP fluorescence and that individual colonies often had different mean fluorescence intensities. After subtracting autofluorescence and calculating the mean of the distribution for cells from each tested colony, we found that most of the transformants shared the same mean value, but some colonies had mean fluorescence values that were integer multiples of the lowest mean value. This finding was consistent with the idea that colonies with the lowest mean value had one copy of the fusion gene, those with twice this value had two copies, and so on. Therefore, in each step of strain construction, we selected as single copy strains those that showed expression at the lowest mean value, except in the case of TCY3216, where we chose a strain that seemed to have a double integration at the ura3 locus, four copies total, because the parental strain, TCY3057, already had two copies of YFP-STE5.

1.3 Plasmids

We first made p_{P_{STE5}-YFP-A206K-CEN}, a single-copy URA3-marked CEN ARS plasmid containing the STE5 promoter, which we defined as a region spanning 1.0 kb upstream of the first codon of the STE5 coding sequence, followed by the YFP-A206K
YFP derivative. We introduced the A206K mutation into \( p_{\text{STE5}} \)-YFP-CEN \(^5\) to reduce the formation of YFP dimers that may alter the biological activity of proteins fused to YFP \(^7\). We then constructed \( p_{\text{STE5}} \)-YFP-STE5-URA3 and \( p_{\text{STE5}} \)-YFP-STE5-TRP1 plasmids in two steps. In the first step, we constructed \( p_{\text{STE5}} \)-YFP-STE5-URA-CEN by homologous recombination in yeast. To do that we co-transformed a \( \Delta \text{ste5} \) ACY379 derivative with \( p_{\text{STE5}} \)-YFP-A206K-CEN linearized downstream of the YFP sequence with SexAI and XhoI, and a PCR product containing the full coding sequence of the STE5 gene plus 44 nucleotides corresponding to its 3'UTR generated using primers which contained 40 nucleotide-long tails homologous to the ends of the linearized plasmid. We selected recombinants on uracil deficient plates and screened for colonies that arrested cell division when treated with \( \alpha \) factor. Since the parental strain was \( \Delta \text{ste5} \), only strains carrying plasmids with a functional STE5 gene responded to \( \alpha \) factor. We then recovered the plasmids from yeast and confirmed that the fusion was correct by sequencing. In the second step, we subcloned a BglI fragment from the recovered \( p_{\text{STE5}} \)-YFP-STE5-URA-CEN containing \( P_{\text{STE5}} \)-YFP-STE5 into the integrating plasmids pRS406 and pRS404 cut with BglI to yield \( p_{\text{STE5}} \)-YFP-STE5-URA3 and \( p_{\text{STE5}} \)-YFP-STE5-TRP1.

To construct \( p_{\text{GAL1}} \)-YFP-A206K and \( p_{\text{GAL1}} \)-YFP-A206K-ADH1tail, we introduced the A206K mutation by site-directed mutagenesis into \( p_{\text{TC7}} \)-YFP and \( p_{\text{TC7}} \)-YFP-ADH1tail (single-copy URA3-marked CEN ARS plasmids in which \( P_{\text{GAL1}} \) directs the synthesis of YFP \(^5\) or YFP-ADH1tail, respectively). We made \( p_{\text{TC7}} \)-YFP-ADH1tail by subcloning an EcoRI/NotI fragment from \( p_{\text{ACL7}} \)-YFP \(^6\) into \( p_{\text{TC7}} \). \(^5\) We finally removed the CEN-ARS to make them into integrating vectors (\( p_{\text{TC7}} \) plasmid series have their CEN-ARS flanked by AatII restriction sites \(^5\)).

To construct \( p_{\text{ACT1}} \)-YFP-A206K and \( p_{\text{ACT1}} \)-YFP-A206K-ADH1tail, we replaced \( P_{\text{GAL1}} \) from \( p_{\text{TC7}} \)-YFP-A206K and \( p_{\text{TC7}} \)-YFP-YFP-A206K-ADH1tail with \( P_{\text{ACT1}} \) (in this case, defined as a stretch of DNA spanning 1.2 kb upstream of the first ATG of the ACT1 gene) by homologous recombination in yeast. To do that, we co-transformed ACLY379 cells with \( p_{\text{TC7}} \)-YFP-A206K or \( p_{\text{TC7}} \)-YFP-YFP-A206K-ADH1tail cut with Acc65I/EcoRI and a PCR product containing the \( P_{\text{ACT1}} \) generated with primers with 40-nucleotide-long tails with homology to the ends of the cut plasmid, and selected colonies on uracil deficient plates. We recovered recombinant plasmids from these cells, and checked them by direct sequencing. We finally removed the CEN-ARS to make them into integrating vectors. \( p_{\text{ACT1}} \)-YFP-A206K and \( p_{\text{ACT1}} \)-YFP-A206K-ADH1tail direct the synthesis of YFP mRNAs with very similar nucleotide sequence. However, the stop codon present at the end of the YFP gene in \( p_{\text{ACT1}} \)-YFP is absent in \( p_{\text{ACT1}} \)-YFP-ADH1tail, and thus the YFP-Adh1tail protein contains a 15 residue C terminal linker and a portion of the 3' end of the ADH1 gene. See Supplementary Figure 2 for further details.

### 1.4 Western blot quantification

To quantify the numbers of YFP-Ste5 molecules, we identified and eliminated many sources of quantitative error in commonly used western blotting procedures \(^1,8,9\). The
key elements of this approach, which will be detailed elsewhere (Benjamin et al., in prep), included quantitative lysis of cells and extraction of proteins, minimization of protein loss on plastic tips and tubes, loading of 3-6 different amounts of each sample, and use of a secondary antibody covalently linked to an infrared fluorophore rather than an enzyme. Here, we grew yeast cells in SD medium to approximately 5-10 x 10^6 cells/ml, froze cell pellets (5-10 x 10^7 cells) in liquid nitrogen, and stored them at –80°C until use.

We prepared total cellular extracts of denatured proteins according to the method of Yaffe and Schatz. Briefly, we resuspended a pellet in 300 µl of 1.85M NaOH, 7.4% β-mercaptoethanol and incubated for 10 min on ice, then added 300 µl of 50% (w/v) trichloroacetic acid and continued incubation for 10 min on ice. We pelleted the precipitate by centrifugation in a 4°C microfuge for 2 min at maximum speed, washed twice with 1 ml acetone at −20°C, dried briefly, and resuspended in gel loading buffer (30 mM Tris base, 50 mM Tris-HCl (pH 6.8), 1.25 mM EDTA, 50 mM DTT, 1% SDS, 10% glycerol, 0.1% bromphenol blue). We loaded four amounts of each protein extract (1-15 µl) into wells of a precast 4-15% gradient polyacrylamide gel (Criterion, BioRad) and electrophoresed at 150-200 V using standard Tris-glycine buffer.

We blotted the gel and probed the membrane with antibodies according to standard procedures. We used a PVDF membrane (Immobilon P, Millipore), and transferred proteins to the membrane by electroporation using a Criterion blotter (BioRad) and transfer buffer containing 39 mM glycine, 48 mM Tris base, 0.01% SDS, and 20% methanol. We used the primary antibodies JL-8 monoclonal mouse anti-GFP (BD Biosciences, 1:1000 dilution) and guinea pig anti-actin (gift of J. Mulholland, Princeton University, 1:5000 dilution). We used secondary antibodies covalently linked to infrared fluorophores, Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, 1:3000 dilution) and IRDye800 goat anti-guinea pig IgG (Rockland Immunochemicals, 1:3000 dilution). We scanned and quantified fluorescence on the membrane using the Odyssey scanner and software (Li-Cor Biosciences).

1.5 Quantification of numbers of Ste5 molecules in living cells with fluorescence

To quantify the numbers of Ste5 molecules in live cells, we compared fluorescence measurements of the YFP-Ste5 TCY3056 strain, in which we swapped out the wild type STE5 gene and inserted one copy of the wild type promoter driving YFP-STE5, using fluorescence from a known concentration of YFP. We measured that these cells contained 434±34 Ste5 molecules on average, which agrees well with our estimate of 484±60 molecules determined by western blotting. This technique is described in more detail in Benjamin et al, in prep.

2 Image Analysis and Data processing
2.1 Automatic focus on the imaged cells

We automatically focused a given field of cells immediately before image acquisition. For fluorescence images in general, many measures of image contrast...
produce a maximum at the stage height corresponding to the focal position. However, the
use of fluorescence for autofocusing can significantly photobleach fluorophores,
including the ones we use for cytometric measurements, and it can be phototoxic\textsuperscript{12}. We
discuss the disadvantages of using fluorescence to find cells in the main text of this paper
and in the next section below. To avoid using fluorescence, we supplemented MetaMorph
with a routine (BF-Focus 1.0) that determined the stage height corresponding to the focal
position. The autofocus routine was written as a Visual Basic subroutine for MetaMorph
5.0 (Universal Imaging, Downingtown, PA) to be called as part of its control of the
microscope stage and camera. In developing the BF-Focus algorithm, we first attempted
to find the focal plane by identifying the z-position of the stage that gave a maximal value
for any one of several measures of image contrast\textsuperscript{11}. We found that the bright field
images showed two local maxima as a function of stage position, one that occurred
slightly above the “true focal plane”—the focal plane that would be found by a human
observer—and one slightly below, and that if we used a definition of contrast as a criterion
for finding the focus, the stage would be set randomly at either of those two stage
positions that exhibit local high contrast above or below the "true focal plane. To address
this problem, we wrote BF-Focus to search for the local minimum in contrast between
those two local maxima. We set the BF-Focus 1.0 routine to dynamically choose the focal
plane each time the motorized stage brought an image field over the objective at different
times. We verified by human inspection that the z-position chosen by BF-Focus yielded
focused bright field and fluorescence images. For the purposes of automated cell
identification by Cell-ID (see below) we captured a bright field image that was
intentionally slightly out of focus, by instructing Metamorph to move the z-position down
150 nm before opening the shutter.

We note that any focus method that relies on the image field itself to determine the
focal position may determine a different position depending on the distribution of cell
sizes in any given image. However, our cells were relatively uniform in height, with the
standard deviation of the average height of the surface area of the cells was 0.65 µ, and
we determined that the uncertainty caused by this effect, given that we typically had at
least \( \sim 20 \) cells in a field of view, is less than the depth of focus of our microscope
objective.

2.2 Identifying Single Cells in Bright Field Images with Cell-ID

It is common for microscope based cytometry methods to use fluorescence to identify
cells. For such cases, edge-detection algorithms, applying a global threshold to the image,
or applying a watershed algorithm (described below) can provide good initial separation
between the fluorescent cells and the non-fluorescent background\textsuperscript{13-17}. We chose not to
use a fluorescence image to identify the cells for our quantitative studies for several
reasons: a) brighter cells would have a higher probability of being scored as cells, b)
because of the microscope optics, some of the fluorescence signal gets spread to regions
of the image near the cell but outside the cell boundaries, and brighter cells produce a
larger signal above background in these regions and thus appear larger, c) heterogeneity
in the intracellular distribution of the fluorescence signal could result in incorrect
boundary assignments, d) the requirement for use of fluorescence channel for cell
labelling reduces the number of other fluorescence channels available, and e) the fluorescence from the molecule used to score cell boundary signal can often bleed into the signal channel. In a recent paper\textsuperscript{5}, we give an example in which the quantitative measurement of fluorescent reporters would have been distorted by bleaching and bleedthrough had we used fluorescence to identify cells. Further examples of measurements of weak signals that potentially would have been obscured and complicated by a fluorescence marker include studies that we have performed on the rates of loss of fluorescence resonance energy transfer (FRET) between regulatory proteins in the nucleus (Yu et al., in prep), as well as rates of re-localization of fluorescent proteins in response to a cellular stimulus (Colman-Lerner et al, in prep.).

A number of methods have been developed that utilize non-fluorescent images to find cells. For example, the “watershed algorithm”\textsuperscript{18} treats the pixels in the image as representing the height of a landscape and then considers how that terrain would fill with water. As the water level rises, higher features become isolated and thus can be separated from the lower background. The method can also be applied fruitfully to local regions of the image since two cells which might mistakenly be identified as one cell will often appear as separate islands before being submerged below the “water”\textsuperscript{13}. An example of its use in non-fluorescently tagged cells can be found in\textsuperscript{15} where the investigators applied it to differential interference contrast (DIC) images of rat adenocarcinoma cells. Another method to identify cells is “active contours,” where the investigator attempts to fit a number of individual closed curves to mark the boundaries of the cells in the image data\textsuperscript{19, 20}. In this method, user intervention is sometimes necessary in the first image of a time course, but, after that, the active contours provide a natural way to track a cell as it changes its shape over time. Other boundary identification algorithms have also been developed. For example one algorithm searches for small regions of an image (of 3T3 fibroblasts) that are significantly above the background level and then “grows” those regions by adding points at the boundary region until no new points above the background level can be added to the region\textsuperscript{21}. Another method scores cell boundaries by first approximating cell boundaries through an analysis at each pixel of the variance of the grayscale values of neighboring pixels, where higher variance is characteristic of pixels located at the boundaries\textsuperscript{22}. The researchers using this method then followed this with a second step in which they sought to apply a threshold to the approximate region to separate the interior and exterior of the cell. It is worth noting that all of these different algorithms (including ours) will sometimes require tuning. For example we found that using a standard Watershed plug-in to ImageJ produced significantly over-segmentation on the bright field images. This is the result of using the algorithm “out-of-the-box,” and other image processing steps could improve the results. We have also found similar results with several “active contours” plug-ins. By contrast, the open source MatLab program CellProfiler (http://www.cellprofiler.org) was able to find cells in bright field images with high efficiency. It does not currently track cells, but our cell tracking algorithm presented below, for example, as well as the algorithm that allows single cells to move slightly between bright field and fluorescence images and a number of the other algorithms also presented below, including the volume measurements and total fluorescence calculations, could be used to fruitfully extend CellProfiler.
For Cell-ID, we divided the process of extraction of single cell information from microscope images into three steps. The first was the identification of single cells in the time series of bright field images; the second was the calculation of various statistics related to each cell, including information from fluorescence images; and the third step was the analysis of the data, including visualization of multidimensional data and data reduction. We performed the first two steps with the Cell-ID program, and the last with CERN’s program PAW (http://paw.web.cern.ch/) in conjunction with a variety of scripts we wrote for use with that program (section 2.3 below).

We wrote Cell-ID in C using the GNU C compiler version 3.2.2. Cell-ID used bright field images to find the cells. Typically, in our bright field images, the border regions of the cells were darker than the rest of the image, and this feature was the primary image attribute that Cell-ID used to find the cells. In order to enhance the darker border regions, we moved the stage 150 nm down so that the image was acquired slightly above the "true focal plane" as described above. This produced a greater contrast for the bright field image. For the fluorescence images, the program returned the stage to the "true focal plane." To identify the greyscale value that corresponded to the boundary pixels, Cell-ID produced a histogram of the greyscale values for every pixel in the image (Supplementary Figure 1a); thus, for a 512x512 image, it produced a histogram with more than 250,000 values. Even for images with many cells, the histogram showed a narrow peak (indicated by the high peak region in the log-scale plot of Supplementary Figure 1a) and a low-end tail. The low-end tail contained the darkest pixels, which included the cell boundaries. Cell-ID defined boundary pixels to be those below a user-defined threshold. In these experiments, we typically chose the threshold to be the mean of the distribution less 1.5 standard deviations ($\sigma$). We generally found that anywhere from 0.5 $\sigma$ to 3.0 $\sigma$ gave good results; but, as the value got smaller, the probability increased that the program misidentified local variations and random fluctuations in the background level of the image as cells. These false cells were easily rejected in subsequent “offline” analysis because, in general, they were never tracked through more than one image in a time course, they did not have a fluorescent signal above background associated with them, they were irregularly shaped, and the boundary region of the false cells was generally near the background level of the bright field image while for real cells the boundary region was significantly darker.

The next step in locating the cells was to find sets of contiguous bright pixels surrounded by these dark boundary pixels. Cell-ID did this by creating sets of contiguous pixels whose values were above the threshold. The boundary pixels between these sets thus corresponded to the boundary pixels of the cells. Cell-ID generally found one or two very large sets (100,000 or more pixels), which were the pixels from the background of the visual field. Yeast cells in the image showed up as smaller sets of contiguous pixels, on the order of 100 to 1000 pixels. A user-defined parameter allowed control of the minimum and maximum number of pixels allowed for a cell. The default lower and upper values of 100 and 1500, respectively, generally gave good results for exponentially growing yeast cells, although the maximum would of course need to be raised for images of larger cells such as most higher eukaryotic cells. For each set of contiguous pixels
whose size fell within these ranges, Cell-ID found the boundary region of the set, and all the pixels inside that boundary were labelled as a cell.

The program then examined each of the scored cells to determine if the scored cell might actually be two cells that were close together. To do this, the program calculated a statistic that used only the boundary pixels. For every combination of two pixels on the boundary, Cell-ID calculated both the distance along the boundary path and the Euclidean distance between them. We found the two points on the boundary for which the value of the boundary path distance divided by the Euclidean distance was at a maximum. This maximum value was larger for cells with a “figure-eight” shape that were pinched in some part than for circular cells. If this maximum was above a user-defined value (which defaulted to 6), then we split the cell into two cells at the location of the pinch (Supplementary Figure 1b). An example of a split cell is shown in Supplementary Figure 1c.

This procedure worked well to split cells that had been incorrectly grouped together, and also to score buds as new cells once the buds became large enough. However, we sometimes had images where the cells had large mating projections ("shmoo tips"; Figure 1), and we found that this procedure would sometimes incorrectly cut off a shmoo tip. To prevent this truncation, Cell-ID performed a second calculation. It calculated the length of the minor axes of the two new cells and compared these lengths to the Euclidean distance between the two pixels at the pinch location, where the original cell was split. If the Euclidean distance divided by the length of the minor axis was greater than a user-defined value (which defaulted to 0.5), for either of the new cells, then the two cells were re-grouped as a single cell. Thus, we required that the two new cells had a generally circular shape and were not too elongated, as would be the case if one of the putative cells was not a cell but a mating projection.

After it found all the cells in an image, Cell-ID gave each of the cells an identification (ID) number and tried to maintain the unique ID number for each cell through the bright field images taken at different times. To track the cells through bright field images of the same visual field taken at different times, Cell-ID first compared each newly found cell to all previously found cells in other bright field images in the time course and quantified the geometrical overlap with all previous cells. We quantified the overlap between two cells in different time frames as the ratio of the number of pixels in the intersection of the two cells divided by the number of pixels in the union of the two cells. Non-overlapping cells had an overlap value of zero, while cells in different images that overlaid each other perfectly had an overlap value of one. For each cell in an image, Cell-ID found the cell in the previous image that produced the highest value for this overlap statistic. If this highest value was above a user-defined cut off (which defaulted to 0.2), the new cell was given the ID number of the previously found cell and was thus identified with that cell in the time course. If no overlap was found, the cell was given a new, unique ID number. Each cell found in every image was uniquely identified by its frame number in the time course and by its ID number.
In this way, the program tracked cells through the time course and also allowed for new cells to appear in the middle of the time course (which could happen if a cell that had been suspended in the culture medium settled on to the glass well bottom in the middle of the time course or if a new cell was produced by budding and cell division), and old cells could disappear (which could happen if cells were shaken loose from the glass surface due to the motion of the motorized stage or changing of the growth medium). Cell-ID created a copy of the original bright field image and annotated the copy by marking the boundaries and ID numbers of all found cells. Viewing the annotated copy allowed us to quickly evaluate how well the program was scoring and tracking cells, and it also allowed us to further examine visually any given cell after the program had finished.

We provide an example of the versatility of this program below where we compare our results to data acquired with a confocal microscope. There we used the Cell-ID algorithm to find the cells in confocal bright field images (for the total fluorescence comparison below), and with slight modification, to find cells in the fluorescently labelled plasma membrane data (for the volume comparisons below). Similarly, we were able to identify mammalian lymphoid cells (HL-60 cells, as mentioned in the main text) without altering the code, and we have also observed that the ability to identify yeast cells is independent of growth conditions, the container in which we grow the cells or perform the imaging, or the strain of yeast.

The implemented Cell-ID algorithm should be able to readily find cells which are regularly shaped and produce dark outlines in bright field images. For some cell images a different cell-finding algorithm might need to be implemented. Cell-ID should also be applicable to different image types, such as phase contrast, differential interference contrast, or even fluorescence images, although for the last case our work suggests that a pre-processing step might be necessary to determine the boundaries of the fluorescent regions.

2.3 Analysis of the statistics with PAW

Cell-ID created a text file containing all the calculated information for all the cells found in all the time frames. We then used CERN’s open source statistical analysis and plotting package, PAW (http://paw.web.cern.ch/paw)\textsuperscript{23}, to analyze this data. PAW is used widely by the high energy physics community and provides a number of features invaluable to analysis of large, multivariable data sets. These include the ability to visualize statistical distributions, including one-, two-, and three-dimensional projections of the data, the ability to easily write and apply potentially complex selection functions to create subsets of the data for further analysis, and the ability to fit the data to user defined models. We used PAW to explore and analyze relatively large, high dimensional data sets that we created by associating large numbers of individual cells with many different measurements. First we reformatted this output text file into a PAW-readable database file. PAW allowed us to readily create complex filters on the data (potentially involving many variables), so that, for example, we were able to exclude putative cells from the analysis if they were too big or too small, if they were irregularly shaped, if their boundary region was too bright in the bright field image, if they failed to appear in all the
images of a time course, or if their fluorescence was consistent with the background. PAW facilitated the creation and plotting of new variables and functions of the variables, and allowed us to construct various kinds of plots in order to help analyze the multidimensional data set. PAW also had a built-in data reduction package, as well as an interface to a FORTRAN interpreter, which allowed us to extend its capabilities as needed.

The routines written for PAW included the calculation of the median of a set of data, the robust calculation of a covariance matrix for two dimensional scatter plots \(^{24,25}\), and the calculation of statistical uncertainties for potentially complicated quantities using a bootstrap technique \(^{26}\). We also used PAW to generate Monte Carlo based simulations, such as the simulation described below to calculate the effect of fluorophore emissions from out-of-focus regions of the cell. All the PAW routines that were used to support this work are available on request.

We considered but did not use two other open source statistical analysis and plotting packages: ROOT (http://root.cern.ch) and R (http://www.r-project.org). ROOT was released by CERN and contains the functionality of PAW but is based on the object oriented language C++, as opposed to PAW which is based in Fortran and PAW’s own scripting language. R is sponsored primarily by the Free Software Foundation (http://www.fsf.org/). This program, like PAW, includes powerful data reduction routines and also has the ability to make plots of one variable after applying conditions to potentially complex functions of other variables (http://CRAN.R-project.org/doc/FAQ). We believe that both of these programs also have the functionality to support analysis of microscopic cytometric data after its acquisition and initial analysis by Cell-ID.

2.4 Total fluorescence with Cell-ID

Once all the cells were found in a bright field image, Cell-ID examined the pixels associated with each cell in the fluorescence images as described below to calculate the total fluorescence and statistics related to the internal structure of the fluorescence. Cell-ID repeated these procedures for each of the different fluorescence images associated with a given bright field image.

To calculate the fluorescence associated with individual cells, Cell-ID superimposed the cell boundaries from the bright field image onto the corresponding fluorescence image. In locating the pixels associated with a given cell in the fluorescence images, Cell-ID allowed a slight (a few pixels) overall shift in the entire fluorescence image to account for small errors in the repositioning of the XY stage between the images. For experiments in which we did not attach cells with conA, the cells sometimes moved slightly during the time between the bright field image and the fluorescence image. To correct for this, Cell-ID took the sets of pixels associated with each cell in the bright field image and allowed them to move slightly in the X and Y directions before examining the corresponding pixels in the fluorescence images. The program maximized the amount of fluorescence within each of the sets of pixels and simultaneously prevented any of the cells from overlapping. It did this by moving each cell one pixel in the
direction of having more fluorescence inside the cell as long as there was no other cell preventing this movement. It then iterated this procedure multiple times. Cell-ID annotated a copy of the fluorescence images with the adjusted cell boundaries, and we verified by eye that this procedure worked correctly. After examining and annotating the fluorescence image, Cell-ID returned the cells to their original position as found from the bright field images. A user-defined parameter controlled whether Cell-ID performed this "wiggling" of the cells in examining the fluorescence images.

As a first approximation, we calculated the total fluorescence as the sum of all the pixel values in the fluorescence image associated with the cell, with the background level subtracted out. We defined the background level to be the mode of the pixel values of the fluorescence images for those pixels not associated with any cell. We used the mode instead of the mean to reduce the impact on the background measurement of fluorescence originated in fluorophores located in out-of-focus regions of the cells that might land in pixels not associated with any cell. We then performed a series of corrections described in the sections below to further refine our fluorescence measurement. In this section we compare these refined measurements with equivalent measurements obtained with a confocal setup and a state of the art flow cytometer.

We compared our measurements to the sum of fluorescence from image stacks performed with a confocal microscope (Zeiss LSM 510, 488 nm excitation laser at 45.6% power through a 63X/1.4 NA oil immersion DIC lens, and viewed through a 500-550 nm band pass filter with cells attached to the bottom of glass bottomed wells). We examined strains that had multiple (0X, 1X, 4X) copies of the Ste5 promoter driving YFP-Ste5. As with our non-confocal microscope, YFP fluorescence quantitated from images obtained with the confocal microscope increased linearly with YFP-Ste5 copy number, which indicated that our microscope measurement was correctly measuring all the light in the cell. We also observed that the confocal microscope produced more overlap between the single cell distributions for the 0X and 1X strains. This might be due to the fact that the confocal was not necessarily tuned to match our optical setup (for example, the emission band-pass filter was wider than the one we used in our microscope). A complete confocal scan of a single image field typically took several minutes, significantly limiting the minimum time scale of events quantifiable by this means as well as the rate at which new cell field images can be captured.

To gain a better idea of the relative sensitivity of measurements made with our optical methods, we also compared them to those made with one of the most sensitive current flow cytometers, a Beckman-Dickinson LSRII. To do so, we treated cells that contained the α factor-inducible PRM1 promoter driving the expression of YFP (P_{PRM1}-YFP) \(^5\) with different concentrations (0 to 40 nM) of α factor for 60 minutes as described in section 1.1. We calculated fluorescence of individual cells from microscope images as described above, and, in parallel, measured the fluorescence for the same populations using the flow cytometer (Figure 3). The shapes of the fluorescence distributions agreed well at all doses tested and the median response at each dose matched reasonably well (Figure 3, inset). However, at very low doses of α factor, a treatment that induced the synthesis of small numbers of fluorescent protein molecules and resulted in total
fluorescence near the level displayed by untreated cells, the microscopic methods resulted in more accurate discrimination between different doses of α factor, and between treated and untreated cells (Table 1).

2.5 Using time course data to correct total fluorescence

We used time courses of volume measurements for each cell to refine measurements of total fluorescence. The measurements of total fluorescence depended on the boundaries drawn for each cell, but the boundaries were subject to some measurement error because the images were pixelated, and determination of the precise boundary was sensitive to small changes between images. Boundary variations correlated strongly with variations in measured volume, and, therefore, to help correct the fluorescence measurement for errors in the boundary determination, we took advantage of the fact that the calculated cell volume at a given time $V(t)$ was described well by the exponential growth function

$$V(t) = V_0 \times e^{gt}$$

where $V_0$ is the volume at time 0, and $g$ is the growth rate. We fitted the volume measured over a time course to this function, calculated a correction factor (the fitted value for $V(t)$, divided by the value of volume that Cell-ID reported at that time point based on the bright field image), and scaled our measured fluorescence by this correction factor.

2.6 Photobleaching correction to total fluorescence

We measured the rates of photobleaching of YFP and CFP caused by excitatory light during image acquisition and used these rates to correct for photobleaching. To calculate these rates, we included in every time course experiment an internal control, which we have mentioned previously but describe in detail here. For the internal control, we captured an extra fluorescent image for one of the visual fields, so that that field received twice as much excitatory light as the others. We compared the fluorescence from cells in this field to that from other fields in the same well that had been imaged only once per time point. From this information we calculated the photobleaching rate and used it to correct the measured fluorescence in each time point. For our mercury arc lamp illumination setup, we typically found that on average 6% of the YFP intensity inside cells for a 1 second exposure disappeared between sequential images suggesting that this proportion of the fluorescent molecules were photobleached with each acquisition. We also measured the percent reduction for single cells, and we found that under identical microscope conditions the single cell distributions were typically Gaussian distributed about the mean with a Gaussian width of 2%. We compared a time course of single cells, and we did not see any correlation between the measured photobleaching for a given cell at one time point with the measurement for the same cell at a later time point, just a few minutes later. This implied that the single cell spread in photobleaching was primarily due to measurement variations. Moreover, at each time point the average percent reduction of the distribution of single cells produced means that varied between about 4% and 8%.

For CFP we typically found that an average of 16% of the CFP intensity inside cells (for a 0.1 second exposure) disappeared between sequential images. We also typically
found that an additional 0.1 sec exposure with a CFP filter cube increased average YFP photobleaching from 6% to 9%.

2.7 Point spread function and correction for out of focus light from the cell

Light that originated from regions of yeast cells above or below the focal plane could be significantly out of focus, and thus, at the surface of the CCD, fall outside of pixels assigned as the borders of the cell. The further away from the focal plane that a photon originated, the less likely it was that it would fall within that boundary. A common procedure is to attempt to use the PSF information to deconvolve the images for the effect of this blurring. Deconvolution in general is an “ill-posed” problem in which, for example, small levels of change in input can drastically affect the outcome, and, in deconvolution, one often needs to apply some additional algorithm to force the answer towards a physically meaningful result, for example, by requiring that all pixels have positive values. To avoid the need for such intervention, which would have complicated our data analysis, we chose instead to simulate the microscope optics (including the limited depth of the focal plane) and CCD camera and to use the simulation to calculate the amount of light lost due to out of focus regions of the cell. We then devised a correction factor for each cell that depended on the average height of the cell.

To calculate a correction factor for the out of focus regions of the cell, we began by characterizing the illumination levels as a function of stage height as well as the microscope resolution at each height. To do so, we made use of the commonly used technique of imaging small fluorescent beads at different stage heights. Specifically we imaged 0.2 \( \mu \text{m} \) diameter fluorescent beads (Molecular Probes, catalogue number F-8807) (smaller beads were not necessary since each pixel in the CCD images corresponded to a square in the visual field 0.21 \( \mu \text{m} \) on a side), and captured fluorescent images of the beads at Z settings of the microscope that differed by 0.1 \( \mu \text{m} \) increments.

We used these measurements to derive a "point spread function" (PSF), which described how light emitted from a point source located at different Z positions was spreads over the CCD surface. For the fluorescent bead images taken at each Z step, we drew a line through the center of the bead and recorded the light falling on each of the pixels along this line. This set of values for each line of pixels defined a “line scan.” We then applied two relatively small corrections to the line scan data to account for experimental effects that would make the measured line scan wider than the "true" point spread function. The first accounted for CCD pixelation. The pixel size results in the convolution of the PSF with a square wave of width 0.21 \( \mu \text{m} \). For example, an infinitely sharp point spread function will be measured as a square distribution 0.21 \( \mu \text{m} \) wide instead of 0 \( \mu \text{m} \). A square distribution which is 0.21 \( \mu \text{m} \) wide has a variance of 0.0037 \( \mu \text{m}^2 \), and we expect the CCD pixelation to increase the spread of the line scans by roughly this amount. The second effect was the finite width of the beads (0.2 \( \mu \text{m} \)). With an infinitely good PSF, our line scan would simply be a perfect image of the bead, which would sometimes cross the boundaries of more than one pixel. The variance of a half circle of diameter 0.2 \( \mu \text{m} \) is 0.0025 \( \mu \text{m}^2 \), and we make the approximation that the line
scan variances are increased by this amount. We used PAW to fit each line scan to a Gaussian shape and reduced the variance by these two quantities. We then used the resulting curve as the value of the point spread function, PSF(Z), along a line through the center of the light source (Figure S1a). We assumed that the PSF is rotationally symmetric in the plane of the CCD image, and we used the corrected line scans to calculate the PSF at any point in the CCD image plane.

We then counted the total light emitted from the beads, even light that fell on pixels of the CCD far from the bead pixels. The total light was calculated as the sum total of a circular set of pixels centered on the bead and that extended far enough away from the bead that the pixel intensities at the boundary of the region were consistent with the non-fluorescent background. We then corrected the total light for the non-fluorescent background that we assumed was constant over the entire image. We observed that the total captured light did not vary with the stage height, and we concluded from this that the excitatory light uniformly illuminated the entire Z dimension of yeast cells (whose maximum diameter was approximately 6 \( \mu \)m), even those parts of the cells that were not in focus (not shown). We note that the working distance of our lens was around 210 microns, so that a change of 6 \( \mu \)m around the focal position did not cause much change in the light collecting efficiency of our lens. Indeed for our oil immersion, NA=1.4 lens, we calculated that the change in light collection efficiency was from -0.8% to +0.8% of the efficiency at the focal position, as we moved from the lowest Z position analyzed to the highest.

To test our measured point spread function, we compared results from a simulation that used the PSF(Z) with experimental data. To generate the experimental data, we labelled the plasma membrane of yeast cells with a lipophilic fluorescent dye, FM 4-64 (N-(3-triethylammoniumpropyl)-4- (6-(4-(diethylamino)phenyl)hexatrieny1) pyridinium dibromide, Molecular Probes, Eugene, OR). We fixed exponentially growing yeast cells with 2% formaldehyde at 4°C for 1 hour, washed cells twice with PBS, incubated with 10 \( \mu \)g/ml of FM 4-64 for 15 minutes and then washed the dye by two washes with PBS. In this way we obtained cells in which the only source of fluorescence was the uniformly labelled plasma membrane. We imaged the cells using a TRITC filter set (TRITC filter set 31002 Chroma Technologies Corp, Brattleboro, Vermont, excitation centered at 540 nm with 25 nm wide bandpass, dichroic mirror passing above 585 nm, and emission filter centered at 605 nm with 55 nm wide bandpass). We then measured line scans through the center of these cells. To compare the measured line scans to those expected from our PSF, we simulated spherical cells whose centers were located at the focal plane, generated a uniform distribution of fluorescence on the surface (ie "plasma membrane") of each simulated cell, and for each point on the surface, looked up the measured PSF for that value of Z, and used the PSF to calculate what amount of light would appear at each pixel of the CCD, even those pixels not associated with the simulated cell. We defined the pixels associated with a simulated cell as those pixels of the CCD chip that would have shown up in the interior of a bright field image of that cell. We then generated a simulated line scan through this simulated CCD cell image. Figure S1c shows a comparison of such a simulated line scan with an actual line scan obtained from a yeast cell with a fluorescently labelled plasma membrane. This good match between observed
and simulated membrane fluorescence data suggested that our derived PSF accurately predicted the light intensity at the CCD camera that originated from fluorophores located at different positions within a cell.
Figure S1. Point spread function calculation. a. Determination of the PSF function for our microscope setup. We imaged a 0.2 µm diameter fluorescent bead (Cat # F8807, Molecular Probes) at a series of stage heights \(Z\) separated by 0.1 µm over a range of 6 µm. We then measured the fluorescence intensity across a 4 µm line that passed through the center (0 on the abscissa) of the CCD image of the bead, at every value of \(Z\) (“line scan”). Plot shows line scans at \(Z=0\) µm (●, focal plane), 0.5 (▲), and 1 µm (■).
b. Calculation of a correction factor for out-of-focus regions of the cell using simulated fluorescent cells. Top. A simulated uniformly fluorescent spherical cell of radius 2.4 µm. Left. Amount of light that falls within the simulated cell boundaries on the CCD image (x-axis) that originated at the indicated \(Z\) positions (y-axis) for the case of ideal optics (◆, in which light is collected with the same efficiency at every point of the cell), and for the case of our measured point spread function (●, in which regions of the cell with heights away from the focal plane contribute less light to the total signal because the light from those sources is spread out over the face of the CCD camera to regions outside of our measured cell boundaries). The shaded region is the total amount of light lost. Right. Fraction of light (x-axis) originating at the indicated \(Z\) positions (y-axis) retained within the boundaries of the cell image on the CCD camera (◆ and shading in the circle, which represents a cell viewed from the side, where white=1, black=0). Bottom. We simulated cells of varying radii (round circles in plot) resting on the bottom of the well (CS), so that the focal plane (FP) passes through the center of a cell with radius ~2.4 µm. We then calculated the fraction of the total light retained (the total area under the red circles divided by the total area under the blue diamonds in the top left plot) within the boundaries of the cell image on the CCD camera.
c. Test of the PSF function. Line scan (▲) of a cell with its plasma membrane labelled with the fluorescent dye FM 4-64 (N-(3-triethylammoniumpropyl)-4- (6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide, Molecular Probes, Eugene, OR), and a line scan simulated using the PSF function (■). The fluorescence intensity of the simulated scan was scaled to match the actual scan at the peak intensity.
We then used our measured PSF(Z) function to determine how much light was lost in images of fluorescent cells due to the fact that parts of the cell were above and below the focal plane. To do so, we simulated fluorescent cells in which fluorescence was uniformly distributed throughout the interior of the cell (Figure S1b). In this simulation, we treated each cell as a sphere of a given radius \( R \) resting on the glass bottom of the microtiter well. We set the focal plane at a height of 2.4 \( \mu \)m above the glass bottom, roughly the average observed radius of yeast in an exponentially growing culture (not shown). We considered the cell to be a series of cross-sectional slices in \( z \). At each value of \( z \), we calculated how much of the light that originated from that height would hit the simulated CCD image at pixels associated with the interior of the cell image. We did this for the case of “ideal optics”, in which the light was perfectly focused, and again for the case in which the light was spread according to PSF(Z) (Figure S1b, top left panel). We then calculated the ratio of the area under the curve determined using the measured PSF to the area under the curve for the ideal optics case. This ratio is the fraction of light that is included within the boundaries of the cell image after discounting the “lost light” that would fall outside the pixels on the CCD identified with the cell (shaded area in Figure S1b, top left panel). We did this calculation for a number of simulated small, medium, and large cells that might be expected in a random sample of cells (Figure S1b, bottom panel).

Figure S1b (bottom panel) shows the fraction of light included in the cell image as a function of cell radius for cells of different sizes. Again, each cell rests on the bottom of the well and the focal plane is at \( z = 2.4 \mu \)m above the well bottom, so it passes above the centers of small cells and below the centers of large ones. Unexpectedly, the fraction collected is relatively independent of cell size. This is because both the smallest and biggest cells have a larger fraction of the cell out of focus than average sized cells. For the smallest cells, a larger fraction is out of focus because the focal plane passes above the widest region of the cell, for the biggest cells, a larger fraction is out of focus because the focal plane passes below the widest region of the cell (see Figure S1b). The fact that both the smaller and larger cells have similar corrections for out of focus light has the effect of reducing the overall variation of that correction.

In order to correct our actual cells, we began by fitting the calculated fraction of light lost because of out-of-focus regions of the cell, as described above, to a smooth function that depended on the radius of the simulated cells. We tried different functions and determined that a quadratic scaled by an exponential fit the data well. We then used PAW to fit the data to such a curve and determined that the function

\[
 f_{\text{cor}} = \left( -0.57960 + 0.74075 \times R - 0.0072983 \times R^2 \right) \times e^{1.3600 - 0.83636 \times R} 
\]

fits the points in Figure S1b (bottom panel) well where \( R \) is the generated radius of the cell in microns. This curve applies to spherical cells, which are the types of cells we simulated, but we reasoned that for irregularly shaped cells, where there is no obvious quantity to call the “radius,” the quantity to use to determine the amount of light lost to regions above and below the focal plane is the average height of the surface of that cell. A cell, for example, with a large shmoo might not need much correction if it did not
extend much above and below the focal plane, even though it contained a large volume. Because, in general, the average height of the cell surface, even for irregularly shaped cells, is given by one half of the ratio of volume to cross sectional area, we calculated for each cell a value of the “radius” $R$ given by

$$R = \frac{3}{4} \frac{\text{Volume}}{\text{area}}$$  \hspace{1cm} (2)

This quantity has the property that for spherical cells, it is the actual radius of the cell, and for irregularly shaped cells, it is proportional to the average height of the cell. Our procedure to the correct each cell for out-of-focus regions of the cells was therefore to calculate $R$ from equation (2), to use that value of $R$ to calculate the quantity $f_{cor}$ from equation (1), and finally to correct for the out of focus regions of the cell by dividing the total fluorescence value measured in the fluorescence image by $f_{cor}$. Values for $f_{cor}$ for the cells shown in Figure 1 of the main text are listed as “psf” in Supplementary Materials Table 2.

The average correction factor for out of focus light was on the order of 1.7, which is relatively large. However, the variance of the correction divided by the mean squared of the correction, and thus the maximum increase in the coefficient of variation introduced by this procedure, varied only between 0.004 and 0.02, depending on the shape of cells (which change, for example, when we treat cells with $\alpha$ factor). These values are quite small compared to the naturally occurring cell-to-cell variation in fluorescence (defined as the variance divided by the mean squared) (~0.2) that we observed for expression fluorescent reporter genes.$^5$

### 2.8 Correction for nonuniform intracellular distribution of fluorescence

Another challenge in the quantification of fluorescence information from these cells came from the fact that fluorescence was not uniformly distributed. Visual inspection of the images showed that YFP or CFP signal was excluded from some subcellular volumes. The most significant region of excluded fluorescence corresponded to the vacuole. For our fluorescence calculation we simply summed all the pixels associated with a cell and corrected by $f_{cor}$, as discussed above, to account for out of focus regions of the cell. If all regions of the cell were equally in focus, our measured fluorescence would reflect the correct total number of mature fluorophores. The vacuole would create dark regions in the image, but those dark regions would correctly reflect that there were fewer fluorophores in the cell. However, because the point spread function depended on cell height, we realized that cell-to-cell differences in vacuole volume and location could cause our total measured fluorescence to be an incorrect measure of the total number of mature fluorophores. For example, if two otherwise identical cells had the same size vacuole, but one vacuole was located in the focal plane and the other above the focal plane, we would incorrectly infer that there were fewer fluorophores in the cell that had the vacuole in the focal plane.

For bright fluorescence signals and for cells with a high degree of non-uniformity, a research could make use of a confocal image stack to measure the effect of the non-
uniformity on the fluorescence. For our cytoplasmic fluorescence measurements, to
determine the magnitude of this effect, we began by generating uniformly distributed
fluorophores in simulated cells as above. For each generated cell we also created a
spherical region from which we excluded any fluorophores. This spherical region
corresponded to a simulated vacuole (we discuss the size distribution of this vacuole
below). We randomly positioned the vacuole within each generated cell. We then used
the measured point spread function to calculate how much fluorescence would be
observed in the CCD camera for each cell with a randomly located vacuole and divided
that by the total amount of fluorescence that would have been observed if a) all regions
had been in focus and b) we had a point spread function that was much less than a pixel
in width. We then took the additional step of dividing the values thus obtained by the
point spread function correction factor $f_{cor}$, described above. The resulting “vacuole
correction factor” is a measure of the error inherent in our measurement procedure
introduced by existence of the vacuole. This number affected our data by increasing its
spread. That is, the variance divided by the mean squared of the vacuole correction
factor would be the value by which the variance over the mean squared of the data was
increased by the existence of the vacuole.

To determine the distribution of vacuole sizes to use in the simulation, we
examined living cells in fluorescence images to score the vacuole by searching for
contiguous pixels of darker regions, and used these regions to estimate the volume of the
vacuole. We determined from this that the ratio of vacuole volume to cell volume could
be fit to a normal distribution with mean 0.36 and standard deviation 0.1. We distributed
the simulated vacuole sizes based on that distribution, and we distributed the radii of the
simulated cells according to the measured radius distribution in real cells. We found that
the variance divided by the mean squared for the correction factor for the vacuole was
0.0004. This quantity was negligible compared to the values of the variance divided by
the mean squared of the naturally occurring variation in the expression of fluorescent
reporter genes (~0.2)\(^5\). Even when we doubled the width of the vacuole distribution, the
number remained negligible, increasing from 0.0004 to 0.00046. Since these coefficients
of variation are negligible, we did not correct the data for the effect of the vacuole.

2.9 Examples of individual cell statistics

Cell-ID generated a series of statistics for each cell at each time point. For example, these
included the number of pixels associated with the cell, its minor and major axes, its
inferred volume, its circularity, the sum of the value of all the pixels for that cell in the
fluorescence image, the fluorescence in various annuli relative to the boundary pixels, the
location and brightness of the nucleus (in images with fluorescently labeled nuclei), and
the actual time of the image. A subset of the statistics calculated is shown in
Supplementary Table 2, for the cells shown in Figure 1 of the main text. In general, Cell-
ID was easily changed to add new statistics as needed.

As an example of individual cell statistics, we discuss two measurements of
circularity and, in the section below, two measurements of cell volume. In some
conditions we were able to exclude false cells and clumps of cells by requiring that
genuine cells have a particular range of circularity values. These two circularity measurements are called $C_{\text{RATIO}}$ and $C_{\text{FFT}}$. The first measurement uses the fact that a circle is the closed curve that allows the maximum area to be enclosed by a perimeter (or circumference) of given size. Explicitly, we defined the quantity

$$C_{\text{RATIO}} = \left( \frac{P^2 - 4\pi A}{4\pi A} \right),$$

where $P$ is the perimeter and $A$ the area of the cell. This quantity is always greater than 0, and the more circular the cell, the closer the quantity $C_{\text{RATIO}}$ is to 0. The second circularity measure is more complicated. For that measurement we begin by calculating the centroid of the cell. We then calculate the length $D(\theta)$ of the line segment that starts at the centroid point, that terminates on the measured boundary of the CCD image of the cell, and that makes an angle $\theta$ with the x-axis, where we considered 128 values of $\theta$ evenly spaced from 0 to 360°. We reasoned that for a circular cell, $D(\theta)$ would be a constant and that as the cell became more non-circular, $D(\theta)$ would become more and more oscillatory. To quantify this distinction we performed a Fast Fourier Transform $\text{FFT}(\omega)$ on the 128 points at which we evaluated $D(\theta)$ (see http://mathworld.wolfram.com/FastFourierTransform.html). For a circular cell, only $\text{FFT}(0)$ would be non-zero, and as the cell became more non-circular, $|\text{FFT}(0)|$ would become smaller, and the quantities $|\text{FFT}(\omega)|$ for $\omega > 0$ would become larger. We defined

$$C_{\text{FFT}} = \sqrt{\sum_{\omega > 0} |\text{FFT}(\omega) / \text{FFT}(0)|^2}$$

where the sum runs over $\omega > 0$. Like $C_{\text{RATIO}}$ this quantity is 0 for circular cells and rises above 0 as the cells become more and more irregular. For highly irregular cells, our calculation of $C_{\text{FFT}}$ can fail for two reasons: The centroid point may actually lie outside the cell, or there may exist an angle $\theta$ for which a line starting at the center of the cell may pass out of the cell and then back into the cell. For these situations we simply set $C_{\text{FFT}}$ to -1, and we have observed that they generally do not occur for real cells and sometimes occur for irregularly shaped, false cells.

As an example of a subcellular fluorescence measurement, Cell-ID calculated the fluorescence at the cell perimeter and then various one pixel wide annuli inward and outward from the cell perimeter. These measurements are useful for experimental analyses of the translocation of fluorescent proteins to the cell boundary. Another example of a subcellular fluorescence measurement is the identification of the nucleus in cells containing fluorescent molecules localized to the nucleus. In experiments not discussed in the main text, we fluorescently labelled the nucleus, and Cell-ID calculated the location and fluorescence intensity of the nucleus. It first found the location of the nucleus by placing circles of a given radius at all possible positions within a given cell. The program then found the circle which had the maximum amount of fluorescence associated with it. The center of this circle then marked the center of the nucleus in the CCD image. The average pixel intensity within the circle was a measure of the fluorescence intensity of the nucleus. However, while Cell-ID could calculate the fluorescence intensity in the nucleus it could not calculate the nuclear size directly because the spread of the light is strongly affected by the amount of nuclear fluorescence in a given cell as well as the height of the nucleus within the cell relative to the focal plane. We can imagine a similar analysis being applied to the vacuole or other fluorescently labelled intracellular regions.
2.10 Volume calculation with Cell-ID

We devised two methods to calculate cell volume. Both methods make assumptions about the height of the cell surface for every pixel in the interior of the cell image. This is commonly necessary: for example, for coliform bacteria, a common assumption is that the bacteria are cylinders with two hemispherical caps \(^29,30\); or, for budding yeast, Woldringh et al. \(^31\) divided fluorescently stained cells at the bud neck and then treating the future mother and future daughter as two intersecting volumes of rotation. The first method to calculate cell volume we referred to as the “union of spheres”. This method distributes spheres that are centered at every possible pixel on the equatorial plane of the cell as given by the bright field image and whose radii are such that the edges of the spheres just touch the cell boundary. The union of all the pixels in these spheres enclosed a space whose volume approximated the volume of the cell. This method works for spherical cells, budding cells, and shmooing cells without needing to know which of the three categories the cells fall into. The second method we refer to as the “conical” method. In this method Cell-ID began by summing all the pixels in the bright field image of the cell to give the area of equatorial plane. It then removed all the pixels on the boundary and added the remaining pixels to the sum. It repeated this procedure multiple times, removing the outer annulus of pixels and adding the remaining pixels to the running sum, until there were no pixels remaining. The resulting sum was the volume in voxels of a “conical” shape whose base is given by the bright field image. We then multiplied the sum by four since the volume of an ellipsoid is four times that of the conical shape whose base is the equatorial cross-section of the ellipsoid. We found that both methods agreed well with each other. Both the union of spheres and the conical methods give the expected volume for spherical cells (Figure S2 and not shown) and, more generally, for elliptically shaped cells (as calculated from the minor and major axes). For irregularly shaped cells, such as cells with significant shmoos or large buds, they take into account pinched regions (at the bud neck for example), and neither method makes the assumption that there exists an axis of symmetry (as might be necessary, for example, to calculate a volume of rotation). They may thus have a wider range of applicability than methods that calculate the volume of rotation around a symmetry axis.

Figure S2 shows a scatter plot of two quantities for cells treated for three hours with α factor. The x-axis is the circularity measure \(C_{FFT}\) described above. \(C_{FFT}\) increases away from 0 as the cells develop non-circular shapes, as demonstrated by the example cells overlaid on the plot. Since the cells were treated with α factor for several hours, many of the cells developed non-circular shapes (shmoos). The y-axis is the ratio of a naive volume measurement to the union of spheres volume calculation. For this naive volume calculation we took the “radius”, \(R\), of the cell to be \(\sqrt{A / \pi}\) where \(A\) is the cross sectional area of the cell as determined from the bright field image. This is the radius expected for circular cells. We then determined the naive volume as \(\frac{4}{3} \pi R^3\), which is the volume for spherical cells. The plot showed that for more circular cells (\(C_{FFT}\) near 0) the union of spheres volume calculation gave the expected result for spherical cells. It also showed that for non-spherical cells, the naive method over-estimated the volume.
We tested our volume measurements in different ways. First we confirmed that the two methods gave consistent results for all cells, and then we confirmed that each result compared well with the volume of rotation as calculated from the minor and major axes for elliptical cell images. We also performed the elementary task of validating our software by printing out a cell image and measuring the volume of a circular cell image by hand assuming the cell was spherical. We then compared our cell measurements to two independent techniques. We first performed measurements of cell volume using a Coulter Counter (Multisizer 3, Beckman Coulter, Inc., Fullerton CA), which passes cells in an electrolyte solution through a detector that measures changes in direct current impedance and then uses the impedance changes to calculate cell volume (Figure S3a)\textsuperscript{32, 33}. This instrument is thus sensitive to a different set of experimental effects than our image-based measurements. We found good agreement both in the peak location of the distribution as well as the spread of the distribution relative to the mean value, with the exception that the Coulter Counter measurement shows a high-end tail of “cells” of improbably large volume. We visually inspected many microscope images to confirm that these large volumes corresponded to two-cell clumps that Cell-ID had correctly scored as two cells.

![Figure S2. Volume calculation as function of circularity.](image)

Ratio of a naive measure of volume (described below) to the “union of spheres” method described in the text, as a function of the circularity measure $C_{FFT}$ discussed in the Supplementary Material section 2.3, for ACLY387 cells with 20 nM $\alpha$ factor, for 3 hours. Lower values of $C_{FFT}$ correspond to more circular cross sections and higher values to more irregularly shaped cells. The naive volume calculation is the quantity

$$V_a = \frac{4}{3} \pi \left( \sqrt{A / \pi} \right)^3$$

where $A$ is the cross sectional area of the cell as determined from the bright field image by Cell-ID. For a spherical cell, the cross sectional area is given by $\pi R^2$ where $R$ is the cell radius; thus, for spherical cells, we expect $V_a$ to be the correct volume of the cell. The plot shows that for low values of $C_{FFT}$ the ratio approaches 1, as expected if the union of spheres volume calculation works correctly. As $C_{FFT}$ becomes larger, and the cells become less circular, the quantity $V_a$ begins to overestimate the volume. Images of three representative cells for different values of circularity are shown overlaid on the plot. The corresponding data points are highlighted in blue triangles.
We further tested our volume calculation on a cell-by-cell basis by examining image stacks of yeast cells with fluorescently labelled cell walls taken with a confocal microscope (Zeiss LSM 510). For the confocal volume measurements, we used Cell-ID to identify cells using the membrane label in the fluorescence images. We then plotted cross sectional area vs the Z position of the focal plane. We found that these plots fit well to quadratic functions. We fit each curve to a quadratic, and we then integrated the resulting functions over the physically meaningful part of the functions (where the functions are positive), to produce the total volume of the cells (Figure S3b). We found that the volume measurements agreed with the Cell-ID measurements for the same cells within 10%. This level of agreement is consistent with the number of pixels $N$ that Cell-ID associates with a cell having a statistical uncertainty of $\sqrt{N}$, given our cells that have an average of around $N=400$ pixels.

To better estimate the error in our volume measurements because of small fluctuations in the pixels that were identified for each cell from different exposures in a time course, we fitted time courses of individual cells to exponential curves, and we compared the fitted value for the last time point to the measured value at that time point. We found that the difference in these values had a standard deviation of 7.5% of the mean value, and we concluded that repeated measurements of the same cell in different image locations would produce a standard deviation of at most 7.5% of the volume.
These volume measurements are fundamentally simple in that they depend on the geometry of the cells and only two further assumptions. The first assumption is that the bright field image approximates a cross sectional image of the cell, and the second is that the maximum height of the cells is approximately equal to their width. Thus, for example, for a circular cell image, the methods assume that the cell forms a sphere whose radius is given by the radius of the cross sectional image. For an elliptical image, the methods assume that the cell is a volume of rotation about a major axis. The assumptions should hold for example, for yeast cells or bacteria lying on their sides, and for non-adherent mammalian lymphocytes, but would fail for non-circular yeast or coliform bacteria standing on their ends, and cells such a fibroblasts, which are spread out and bulged in the region of the nucleus. However the open source design of the code allows an investigator to easily extend this method to apply to cells with such different shapes. One approach to such an extension would be to scale the radius of the spheroids, for example, depending on the distance from the cell boundaries. One could calibrate and validate this image based volume measurement by calibration experiments using three-dimensional reconstruction of membrane labelled cells from confocal image stacks.

3 Rate calculations
We discuss here several results that make use of our ability to acquire high throughput microscope based fluorescence data. Note that these methods are not dependent on Cell-ID and PAW; if a researcher were to use a different method to measure total fluorescence, such as with a confocal microscope, or perhaps, in the case of the snapshot experiments, a flow cytometer, the methods would be equally applicable.

3.1 Measurement of fluorophore maturation rates
All FPs, including derivatives of *Aequorea victoria* GFP, require a posttranslational reaction to form the fluorophore. For GFP derivatives, fluorophore maturation proceeds through two sequential steps: a relatively fast cyclization of the three amino acids at residues 65-67 followed by a slow oxidation step. This slow step dominates the maturation kinetics, and chromophore maturation effectively follows first order kinetics. Point mutations of the wild type GFP can create FP derivatives with differing characteristics, including excitation and emission spectra, brightness, and maturation time. For example, the S65T mutation leads to a GFP with an extinction coefficient nearly 6 times higher than wild type as well as a maturation time four-fold shorter.

As described in the main text, we examined a yellow FP derivative (YFP) (wt GFP with mutations S65G/V68L/S72A/T203Y) and a cyan derivative (CFP) (F64/S65T/Y66W/N146I/M153T/V163A), in individual *S. cerevisiae* at 25°. This YFP derivative is not as bright as a derivative named “Venus,” but has better stability against photobleaching. A brighter CFP variant (“Cerulean”) was not known at the time we performed many of these experiments, including the experiments in the companion paper.
As described in the main text, we examined single cells in which a pheromone responsive promoter (from the gene PRM1) drove expression of YFP. We treated the cells with high levels of α factor and then added cycloheximide to stop further protein production. After addition of cycloheximide, any further increase in fluorescence would be the result of the maturation of existing YFP. Our data were consistent with the simplest model, in which maturation of the GFP derivatives is dominated by a single step and in which the probability of this step occurring during any small time interval is the same for all times $t$. This is described by the differential equations:

\[
\frac{dI}{dt} = -aI(t) \tag{3}
\]
\[
\frac{dM}{dt} = aI(t) \tag{4}
\]

where $I(t)$ is the amount of immature YFP, $M(t)$ is the amount of mature, fluorescent protein, and $a$ is the rate at which immature protein changes to the fluorescent state. In the presence of cycloheximide, YFP is neither synthesized nor degraded, and thus the total number of YFP molecules does not change with time. The solution to these equations is

\[
I(t) = I_0e^{-at} \tag{5}
\]
\[
M(t) = M_0 + I_0(1 - e^{-at}) \tag{6}
\]

where $t=0$ is the time of cycloheximide addition, and $I_0$ and $M_0$ are the amounts of immature and mature YFP at $t=0$. The average time for half of the YFP molecules to mature, $T_{1/2}$, is $\ln(2)/a$. We corrected each cell for accumulated photobleaching, discussed in Section 2.6 above. We used PAW to fit the data from each cell to the function $M(t)$. This data is described further in the main text.

We then produced distributions of $T_{1/2}$ for thousands of single cells. The distribution had a Gaussian shape with mean 39 minutes and coefficient of variation (CV=standard deviation of the mean) of 0.10. The standard error on the mean was negligible, on the order of 10’s of seconds. The photobleaching correction, however, has a significant effect on the mean of the fit results, and here we estimate the uncertainty introduced from uncertainties on this correction. For example, for these measurements we corrected for 6% photobleaching per image, and if we (incorrectly) include no photobleaching correction, the mean $T_{1/2}$ is reduced to 19 minutes. As discussed in Section 2.6, we typically saw between 4% and 8% photobleaching reduction for fields of cells for 1 second YFP exposures. For a 4% photobleaching correction, we get a mean $T_{1/2}$ of 32 minutes, and for an 8% correction $T_{1/2}$ of 46 minutes. We take these as one standard deviation bounds, and we report a measurement for YFP maturation of $T_{1/2} = 39 \pm 7$ minutes.

For CFP maturation we measured a value of $T_{1/2}=49 \pm 9$ minutes, where, for the uncertainty of 9 minutes, we assumed the fractional uncertainty on the YFP maturation fit would be roughly the same on the CFP maturation fit (9 minutes $\sim 7/39 \cdot 49$ minutes).
We then estimated what part of the cell-to-cell variation was the result of statistical uncertainties from the individual cell fits. To do this we first reasoned that a significant source of uncertainty on the single cell fluorescence measurements was errors in the determination of the exact pixels that make up the boundaries of the cells. We reasoned that this error would depend on the square root of the number of boundary pixels. We estimated that the uncertainty on each cell’s total fluorescence would be the average fluorescence intensity of the boundary pixels scaled by the square root of the number of pixels. We then scaled this quantity by an empirical factor of 0.8 so that the maturation fits discussed above produced an error-weighted sum of squares of the residuals that looked roughly like a $\chi^2$ for the number of degrees of freedom in the fits. We then created simulated data where each simulated cell started with a total fluorescence time course that exactly matched the turn on curve. We then added Gaussian distributed errors to each time point based on the above errors, and we re-fit such time courses many times.

The standard deviation of these fitted results was the expected contribution to the cell-to-cell variation from the statistical errors on the fits. We found that the standard deviation of these fits was 6% of the mean fit result. We assumed that the actual cell-to-cell variation in maturation times was independent of the fitting errors, and we calculated that the actual cell-to-cell variation in maturation times was $\sqrt{(0.1^2-0.06^2)}=8\%$. In the main text, we reported this as CV<10%.

There have been very few quantitative measurements of fluorophore maturation times (See Supplementary Table 1 in [34]) with which to compare our results. In vitro, researchers have measured the slow oxidative step in chromophore formation [34] by treating purified FP extracts with denaturing and reducing agents and observing the recovery of fluorescence as the FP re-folded and re-oxidized upon dilution. These results varied between the extraordinarily fast result of $T_{1/2}<5$ min (for YFP at 37°) [40] and numbers closer to our results of $T_{1/2}=42$ min (for S65T/F64L GFP at 30°) [41], $T_{1/2}=76$ min (for S65T GFP at room temperature) [42], and $T_{1/2}~75$ min (for various CFP and YFP at 37°) [43]. In vivo, Heim et al [38] reported a $T_{1/2}=19$ min in E. coli (for S65T GFP at 22°). These researchers measured the oxidative step by growing cultures anaerobically for 3 days and then tracking fluorescence upon transfer to oxygenated medium. More recently Cross and collaborators reported an effective maturation time $T_{1/2}~15$ min (for S65T GFP at 30°) [44] for an unstable S65T GFP that convolved maturation with protein decay, which makes the maturation rate appear faster than the stable form. We believe it is possible that the first in vitro YFP oxidation study above differed so significantly from our result because of differences between the in vitro oxidative environment and that found in yeast cells. By contrast, the other results are relatively similar. We believe that chromophore differences among the GFP variants, differences in experiment temperature, differences in the amount of dissolved oxygen at the different temperatures, as well as differences between the in vitro and in vivo oxidative environments may contribute to the measurement variation. In any case, the times measured in this study convolve all steps relevant to fluorophore formation in vivo in yeast, including translation, folding, cyclization, and oxidation, under growth conditions which are identical to those used in our experiments and which are similar to those used routinely in yeast experiments.
3.2 Stability of YFP-ADH1tail and GFP-STE5 from galactose shut-off experiments

Although CFP and YFP alone are not degraded significantly, when fused to some peptide sequences they become unstable \(^6,45\). We measured the degradation rate of one of these derivatives, YFP-Adh1tail. This protein contains, C terminal to YFP, a 16 amino acid connecting peptide followed by 60 amino acids derived from the C terminus of Adh1 (Figure 4a and Supplementary Figure 2) \(^6\). We analyzed cells bearing a reporter gene in which the galactose inducible promoter \(P_{\text{GAL1}}\) drove the expression of this fusion protein (Figure 4d). After imaging cells that had been growing exponentially in the presence of galactose, we switched to medium containing glucose, to suppress expression from \(P_{\text{GAL1}}\) \(^46\), and 100 nM \(\alpha\) factor, to halt cell proliferation. We continued to acquire images for 3 hours. In these cells, unlike cells with YFP alone, fluorescence signal from the YFP-Adh1tail diminished over time (Figure 4d).

To calculate the degradation rate, we first determined that, after shutoff of \(P_{\text{GAL1}}\) by addition of glucose, translation from existing mRNA did not significantly contribute to the observed YFP fluorescence (Figure S4). Then, we assumed that the probability of degradation of each YFP fusion molecule was independent of the total number of YFP fusion molecules, and we fit the fluorescence measured in each cell to an exponential decay. At the time of transfer to glucose we expected that around 30% of the fluorophores would be immature (see below), and therefore, to allow these fluorophores some time to mature, we started the fits at 60 minutes after transfer to glucose. We found that waiting a longer time did not significantly change our fit results.

The results of the fit gave an average degradation rate of \(\mu=(3.0 \pm 0.1) \times 10^{-4} \text{ sec}^{-1}\), which gives a half life \((T_{1/2}=\ln(2)/\mu)\) of 39 minutes for YFP-Adh1tail. Within the population, the coefficient of variation \((\sigma/\mu)\) for the fitted degradation rates was relatively small, \(\sim 0.13\), which implies that there is relatively little cell-to-cell variation in the mechanisms that degrade this protein.
We also performed experiments with cells that contained GFP instead of YFP, and in which the inducible GAL1 promoter directed the synthesis of GFP or GFP-Ste5 proteins. We grew these cells on glucose medium and then transferred to galactose medium for more than 24 hours to induce $P_{GAL1}$. We then transferred them to glucose to halt further expression. We collected aliquots every 30 minutes, measured their OD, fixed the cells in paraformadehyde as described and measured average fluorescence concentrations. The rates of decrease in average fluorescence concentration for GFP and GFP-Ste5 after transfer to glucose were similar, and were quantitatively explained by dilution.

3.3 Snapshot experiments: calculation of degradation rates in vivo

Snapshot experiments allow us to infer degradation rates from any measure of total fluorescence, whether obtained by summing a confocal image stack, by means of a flow cytometer, or using the methods described above. To introduce these experiments, we consider fluorescent protein production in a single cell. If the fluorescent protein is produced with rate $\lambda(t)$, and if we assume that there is a constant degradation rate $\mu$ of this protein and a constant fluorophore maturation rate $a$, then, the following differential equations describe the production of immature and mature fluorescent protein, respectively:

$$\frac{dI}{dt} = \lambda(t) - (a + \mu)I(t)$$  \hspace{1cm} (7) $$\frac{dM}{dt} = aI(t) - \mu M$$  \hspace{1cm} (8)

**Figure S4.** After shutoff of $P_{GAL1}$ by addition of glucose, translation from existing mRNA does not significantly contribute to the observed YFP fluorescence. We grew cells expressing $P_{GAL1}$-YFP in galactose medium overnight to induce expression of YFP. Ten minutes before imaging, we attached the cells to ConA coated microtiter plates, and at time 0 we changed the growth medium to glucose medium plus 100 nM $\alpha$ factor (●) or glucose medium plus 20 $\mu$g/ml cycloheximide (▲). In this experiment, we acquired images of the same fields of cells every 10 minutes. Data correspond to the population mean ± standard error of the total YFP fluorescence in each cell. If, after glucose addition at time 0, further translation of $P_{GAL1}$-YFP derived mRNAs were contributing significantly to the total number of fluorescent molecules, then the level of fluorescence of the glucose and $\alpha$ factor treated cells would increase beyond the level of the glucose and cycloheximide treated cells.
where $I(t)$ and $M(t)$ are the amount of immature and mature protein at time $t$. The function $\lambda(t)$ is proportional to the amount of mRNA that is translated into the fluorescent protein.

These equations describe the level of fluorophores in a given cell. To describe the fluorophores in an exponentially growing population of cells, we needed to account for multiple cell divisions, and the fact that every cell inherits part of its complement of fluorophores from its mother. We made the assumption that the population of cells was at steady state, so that the population average of the cellular concentration of protein was constant. We expressed the amount of protein in terms of the concentrations and calculated the population average. We wrote $m(t)$ for the population average of the concentration $M(t)/V(t)$ and $i(t)$ for the population average of $I(t)/V(t)$ where $V(t)$ is the volume of the cell. We assumed that each cell grows exponentially and that the cell population also grows exponentially, with population growth rate $g = \ln(2)/T_{\text{DOUBLE}}$, so that the total volume of the population of cells grows according to $dV/dt = gV(t)$. Using equations (7) and (8) we got

$$\frac{di}{dt} = \Lambda - (a + \mu + g)i \quad (9)$$
$$\frac{dm}{dt} = ai(t) - (\mu + g)m \quad (10)$$

where $\Lambda$ is the population average of $\lambda(t)/V(t)$. The term $\Lambda$ is thus proportional to the population average of the mRNA concentration. The growth rate $g$ behaves in these equations like an additional degradation term, accounting for the dilution of the fluorophores caused by cell growth and division. This kind of analysis is well known: Subramanian and Srienc $^{47}$ derived a similar equation, as did Leveau and Lindow $^{48}$ although the latter treated protein degradation according to Michaelis-Menton kinetics.

At steady state the left hand sides of equations (9) and (10) are 0. We then derived that the population averages for exponentially growing cells was given by

$$i = \frac{\Lambda}{a + \mu + g} \quad (11)$$
$$m = \frac{\Lambda}{(a + \mu + g)(\mu + g)} \quad (12)$$

Similarly, when we assume that the mRNA had a constant production rate $r$ and degradation rate $s$, then the population average of the mRNA concentration of exponentially growing cells was given by

$$\Lambda = \frac{r}{g + s} \quad (13)$$

In the snapshot experiments described in the main text we used these equations to calculate mRNA decay and protein degradation rates in vivo. Explicitly, in the main text we considered the case of two yeast strains each containing a gene encoding a different
YFP-tagged protein, but driven by the same promoter (P_{ACT1} driving the unstable derivative YFP-ADH1tail in one strain and P_{ACT1} driving the expression of YFP in another). We assumed that the mRNA production rate $r$ was the same for the two tagged genes because the promoter regions of the genes were the same. We used equations (12) and (13) to calculate the expected ratio, $Q$, of the YFP fluorescence concentration. The mRNA production rates $r$ cancelled out in the ratio, which was then is given by

$$ Q = \frac{(\mu_2 + g + a)(\mu_2 + g)(s_2 + g)}{(\mu_1 + g + a)(\mu_1 + g)(s_1 + g)} $$ (14)

where the subscripts reference the two proteins. We also assumed that the two mRNA’s had the same decay rates ($s_1=s_2$), and equation (14) became

$$ Q = \frac{(\mu_2 + g + a)(\mu_2 + g)}{(\mu_1 + g + a)(\mu_1 + g)} $$ (15)

Moreover, since YFP is not degraded ($\mu_2=0$), as discussed in the main text, equation (15) becomes

$$ Q = \frac{(g + a)g}{(\mu_1 + g + a)(\mu_1 + g)} $$ (16)

We can then use the measured value of $Q$, $a$, and $g$, to infer $\mu_1$, the degradation rate of YFP-ADH1tail.

We also used the equations to determine the fraction of the fluorophores that are immature, $f_{imm}$, at steady state in exponentially growing cells. This fraction is given by $i/(i + m)$, which we calculated using equations (11) and (12) to be

$$ f_{imm} = \frac{\mu + g}{\mu + g + a} $$ (17)

Thus, $f_{imm}$ is independent of the production or degradation rates of the mRNA. For the case of a stable fluorescent protein ($\mu=0$), a maturation rate $a$ of 0.0003 sec$^{-1}$ and a 90 minute doubling time, we calculated that $f_{imm}=0.3$. 
References