Protocol

Protocol for tracking the inheritance patterns of proteins in live *Saccharomyces cerevisiae* cells using a photo-convertible fluorescent protein

Tracking the inheritance patterns of proteins (TriPP) is a live-cell imaging technique used for tracking maternal protein segregation patterns between mother and daughter cells during asymmetric divisions of budding yeast. We use the photo-convertible fluorescent protein Dendra2 fused to a protein of interest (POI). Irreversible conversion from green to red fluorescence allows for parallel monitoring of old and new proteins for several generations. Single-cell quantitative image analysis of time-lapse microscopy gives synthesis and decay rates, as well as segregation patterns of the POI.
Protocol for tracking the inheritance patterns of proteins in live Saccharomyces cerevisiae cells using a photo-convertible fluorescent protein

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SUMMARY
Tracking the inheritance patterns of proteins (TrIPP) is a live-cell imaging tech-
nique used for tracking maternal protein segregation patterns between mother
doctor cells during asymmetric divisions of budding yeast. We use the
photo-convertible fluorescent protein Dendra2 fused to a protein of interest
(POI). Irreversible conversion from green to red fluorescence allows for parallel
monitoring of old and new proteins for several generations. Single-cell quantita-
tive image analysis of time-lapse microscopy gives synthesis and decay rates,
as well as segregation patterns of the POI.
For complete details on the use and execution of this protocol, please refer to
Auboiron et al. (2021).

BEFORE YOU BEGIN
Why TrIPP?
The inheritance of chromatin-bound proteins theoretically plays a role in the epigenetic transmission
of cellular phenotypes. Protein segregation during cell division is however not systematically studied
and is therefore poorly understood. We now describe a detailed protocol of our method to Track the
Inheritance Patterns of Proteins (TrIPP): a live cell imaging method for tracking maternal proteins
during asymmetric cell divisions of budding yeast. Our analysis of the partitioning pattern of a
test set of 18 chromatin-associated proteins (Auboiron et al., 2021) revealed that most abundant
and moderately abundant maternal proteins segregate stochastically and symmetrically between
the two cells with some notable exceptions-Tup1p, Fpr4p and Rxt3p- that are preferentially retained
in the mother. Low abundance proteins also tend to be retained in the mother cell with the exception
of two tested proteins (Figure 1). This method allows us to analyze the protein behavior in single cells
and the implementation of TrIPP on a large set of proteins should define general trends in cellular
proteome dynamics including the relationship between protein synthesis and decay rates and the
 correlation between protein half-lives and cell-cycle duration. Finally, a TrIPP survey of a large
portion of the nuclear proteome in yeast and other organisms will shed light on different modes
of protein inheritance and open up avenues for research into the role of nuclear proteins in the
epigenetic inheritance of cellular phenotypes.

Preparation of yeast reporter strains with the Dendra2 photo-convertible fluorophore

© Timing: approximately 1 week
1. Construct a strain with the Dendra2 (Shcherbakova and Verkhusha, 2014) protein fused to a protein of interest (POI). The parent yeast strain is transformed with a PCR product from a plasmid carrying the Dendra2 gene and a selection marker using standard yeast transformation protocols (Amberg et al., 2005). The transformed fragment is then integrated at the C-terminus of POI by homologous recombination (Figure 2).

**Note:** In Auboiron et al. (2021), the PCR insert was amplified from the pDendraNatMX6 plasmid that contains the NatMX selection marker for resistance to nourseothricin. The parent strain was JOY1 (MATa ura3Δ leu2Δ his3Δ met15Δ bar1Δ::HIS5, BY4741 background). Integration primers for PCR amplification should contain at least 40 bp of homology sequence with the C-terminus of the target gene and the endogenous stop codon should be excluded. The integration was verified by PCR of genomic DNA with a combination of verification primers on either side of the integration site and within the insert. Integration and verification PCR primers for the HHF2 locus encoding for histone H4 are listed in the Key resources table as an example. If POI is not very abundant (less than 1000 copies per cell), a double or a triple Dendra2 tag should be used instead of the single Dendra2 tag shown in Figure 2.
Stock solutions for media

Timing: 1–4 h

2. Prepare all the stock solutions according to the recipe in materials and equipment.

Note: Stock solutions can be prepared in high volumes and stored indefinitely if they don’t get contaminated. 1x medium should be prepared fresh on the first day of the experiment to avoid contamination. It is very important to use synthetic media to eliminate the green auto fluorescence of the YP medium during image acquisition.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins |
| Yeast Nitrogen Base without ammonium sulfate | Formedium | CYN0510 |
| Ammonium sulfate | Merck Milipore | 101217 |
| Arginine | Sigma | AS131 |
| Histidine | Sigma | H8125 |
| Isoleucine | Sigma | I2752 |
| Leucine | Sigma | L8000 |
| Lysine | Sigma | LS626 |
| Methionine | Sigma | M2893 |
| Phenylalanine | Sigma | P2126 |
| Threonine | Sigma | T8625 |
| Tryptophan | Sigma | T8941 |
| Adenine | Sigma | A8626 |
| Tyrosine | Sigma | T3754 |
| Uracil | Sigma | U1128 |
| Glucose | Sigma | 49159 |
| Galactose | Formedium | GAL03 |
| Nocodazole | Sigma | M1404 |

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# Materials and Equipment

## YNB 10x

| Reagent                                         | Final concentration | Amount |
|------------------------------------------------|--------------------|--------|
| YNB (Yeast Nitrogen Base) without ammonium sulfate | 19 g/L             | 19 g   |
| Ammonium sulfate                                | 50 g/L             | 50 g   |
| ddH₂O                                           | Up to 1 L          |        |
| **Total**                                       | **10x**            | **1 L**|

Filter sterilize with 0.2 μm filters. Keep at 23°C–25°C (indefinitely if not contaminated). Handle using sterile techniques for yeast cell culture.

## DROP-OUT 100x

| Reagent        | Final concentration | Amount |
|----------------|--------------------|--------|
| Arginine       | 2 g/L              | 2 g    |
| Histidine      | 1 g/L              | 1 g    |
| Isoleucine     | 6 g/L              | 6 g    |
| Leucine        | 6 g/L              | 6 g    |
| Lysine         | 4 g/L              | 4 g    |
| Methionine     | 1 g/L              | 1 g    |
| Phenylalanine  | 6 g/L              | 6 g    |
| Threonine      | 5 g/L              | 5 g    |

(Continued on next page)
Note: Adenine, uracil and tyrosine solutions are made separately because of their poor water solubility. Adjust the composition according to the genotype of the used strain.

- **ADENINE 100x**

| Reagent       | Final concentration | Amount     |
|---------------|---------------------|------------|
| Adenine       | 5 g/L               | 2 g        |
| ddH₂O         |                      | Up to 400 mL |
| **Total**     | 100x                | 400 mL     |

Heat at 50°C on a hot stir plate until the powder dissolves and filter sterilize with 0.2 μm filters. Keep at 23°C–25°C (indefinitely if not contaminated).

Note: Crystals can appear after a few weeks at 23°C–25°C. The bottle can then be reheated at 50°C on a hot stir plate until the crystals dissolve. Filter sterilize again.

- **TYROSINE 8x**

| Reagent     | Final concentration | Amount     |
|-------------|---------------------|------------|
| Tyrosine    | 0.4 g/L             | 0.4 g      |
| ddH₂O       |                      | Up to 1 L  |
| **Total**   | 8x                  | 1 L        |

Heat at 50°C on a hot stir plate until the powder dissolves and filter sterilize with 0.2 μm filters. Keep at 23°C–25°C (indefinitely if not contaminated).

- **URACIL 50x**

| Reagent   | Final concentration | Amount     |
|-----------|---------------------|------------|
| Uracil    | 2.5 g/L             | 1.25 g     |
| ddH₂O     |                      | Up to 500 mL |
| **Total** | 50x                 | 500 mL     |

Heat at 50°C on a hot stir plate until the powder dissolves and filter sterilize with 0.2 μm filters. Keep at 23°C–25°C (indefinitely if not contaminated).

- **GLUCOSE/GALACTOSE 20% (10x)**

| Reagent           | Final concentration | Amount     |
|-------------------|---------------------|------------|
| Glucose or Galactose | 20%               | 200 g      |
| ddH₂O             |                      | Up to 1 L  |
| **Total**         | 10x                 | 1 L        |

Filter sterilize with 0.2 μm filters in sterile bottle.

- **COMPLETE SYNTHETIC MEDIUM 1x**
The 1x medium solution is assembled from all the above stock solutions. Note that the final pH should be between 4 and 6 for optimal growth.

Note: Combine all solutions in a sterilized glass bottle and keep at 23°C–25°C. Handle all in sterile conditions (next to a flame or cell culture hood) to avoid contamination. Make just enough 1x medium for the next few days.

### STEP-BY-STEP METHOD DETAILS

#### Culture of Dendra2 strains

@ Timing: 3 days

This step describes the culture of the Dendra2 strain before microscopy.

We assume that colonies have already been grown on a plate (rich medium, Yeast Extract–Peptone–Dextrose (YPD) and Agar) (Amberg et al., 2005). If not, streak cells from a glycerol stock onto a plate and incubate at 30°C for 3 days.

**Day 1**

1. Inoculate 5 ml of synthetic medium with the carbon source of choice with a single yeast colony of a strain with Protein of Interest fused to Dendra2. Incubate overnight (~16hrs) in an incubator shaker at 30°C and 220 rpm.

**Day 2**

2. Measure the OD₆₀₀ of the culture and inoculate 20 mL of the same synthetic medium with the appropriate volume of the overnight (~16hrs) culture so that the OD₆₀₀ is ~0.3 the next day (see calculations below). Incubate overnight (~16hrs) at 30°C and 220 rpm
   a. OD₆₀₀ = 0.5 corresponds to 10⁷ cells/mL, as measured with our spectrophotometer
   b. The inoculation volume of the initial culture needed to obtain a culture with the desired OD₆₀₀ after a defined time interval is calculated with the following formula:

   \[
   \text{OD}_{600\text{final}} \times V_{\text{final}} = \text{V}_{\text{initial}}
   \]

   \[
   2^t \times \text{OD}_{600\text{initial}}
   \]

   with:

   \[t = \text{time of exponential growth (it needs to be adjusted for lag time before exponential growth starts: } t = t_{\text{total}} - t_{\text{lag}} \text{; } t_{\text{lag}} \text{ is typically one generation time so } t = t_{\text{total}} - g\]
g = generation time

Day 3

3. The next day: check that the OD$_{600}$ is around 0.3.

4. Let yeast grow at 30°C, 220 rpm until OD$_{600}$ ~ 0.5–0.8

   **Optional:** Do the following step only if G2/M arrest is needed for the experiment.
   a. Add nocodazole (15 μg/mL final) for 3 h.
   b. Preheat table top centrifuge to 30°C. Spin cells for 2 min at 3000 x g in table top centrifuge with a swinging bucket rotor and remove supernatant.
   c. Resuspend cells in 20 mL of fresh synthetic medium.
   d. Let yeast grow for 45 min at 30°C, 220 rpm.

**Microscopy slide preparation**

© **Timing:** 30 min–1 h

This step describes the preparation of the microscopy slide (Figure 3).

5. Prepare 10 mL of 0.8% agarose in synthetic medium with a desired carbon source, for e.g., glucose or galactose, (0.08 g agarose + 10 mL medium).
6. Warm up suspension in a microwave oven until agarose is dissolved.

   **CRITICAL:** Do not boil the agarose solution to avoid concentrating the solution because of water evaporation.

Do all the following steps next to a flame to avoid contamination.

7. Fill all 8 wells with agarose solution (~350 μL per well) and let the agarose solidify.
8. Use a 10 μL sterile pipette tip to carefully lift the gels and place them upright on the side of the well.
9. Wait a few minutes for the bottom of the well to dry.
10. Measure the OD$_{600}$ of the yeast culture before spinning the entire culture for 2 min at 3000 x g in a swinging bucket rotor of a tabletop centrifuge preheated at 30°C. Remove supernatant and re-suspend the cell pellet in synthetic medium to a final OD$_{600}$ ~ 2.2. This density is optimal for microscopy. Cells are well spaced in the field of vision and their numbers are high enough for statistically significant quantitative analysis. The total number of cells analyzed from five positions in each well and all 8 wells is typically ~300.
11. Pipette 3 μL of re-suspended yeast cells into each well and spread them with the pipette tip.
12. Let dry until there are no more visible liquid droplets on the bottom of the well (10 s to 2 min). This is to avoid “floating” cells during microscopy.
13. Carefully put the agarose gel back down, on top of the cells and avoid trapping bubbles. Cover the plate with its lid.
14. Take your slide to the microscope

**Hi-Lo microscopy**

© **Timing:** 9–12 h

This step describes the microscope settings for optimal yeast growth and image acquisition.
15. Adjust the laser power and LED lamp (Lumencor system) parameters to:
   a. 488 nm laser: \( \sim 360 \text{ mW} \)
   b. 561 nm laser: \( \sim 300 \text{ mW} \)
   c. LED photo-conversion (390 nm): \( \sim 2.30 \text{ mW} \)
   d. Set the temperature of the thermostatic chamber surrounding the microscope stage to 30°C.
   (Figure 4A)

   **Note:** These parameters are optimal for live cell microscopy.

16. Use the following equipment (or equivalent) (Figure 4):
   a. Nikon inverted microscope for epifluorescence and TIRF microscopy in the Hi-Lo mode (60% angle).
   b. Camera EMCCD 512 Photometrics (512*512, 16 \( \mu \text{m} \) pixel size)
c. 60× water objective, NA 1.2 (Nikon)
d. Water dispenser (custom made)
e. Metamorph software

17. Choose 5 positions per well (40 positions in total) and set Z stack parameters (5 stacks separated by 1 μm between each pair).

18. Take one image at each position in the LED Trans mode and at 488 nm and 561 nm excitation wavelengths before photo-conversion. Dendra2 emits green (488 nm excitation) or red (561 nm excitation) light before and after photo-conversion, respectively.

Note: The excitation wavelengths have to be adapted to the type of photo-convertible fluorescent protein used.

19. Photo-conversion is done by exposing cells at each position for 1 min to 390 nm UV light from the LED Lamp at 60% capacity with Neutral Density filters ND4 or ND8. The photo conversion process for all positions in all 8 wells takes ~40 min. The efficiency of photo-conversion can vary from cell to cell but it is mostly greater than 50%. See problem 3 in the troubleshooting section on how to maximize photo-conversion efficiency.

20. Set-up automated time-lapse image acquisition:
   a. For each position, take one photo each in the LED Trans mode, and at 488 nm and 561 nm excitation wavelengths (200 ms exposure) with GFP and TAMRA emission filters, respectively. We use a camera gain of 300 for abundant proteins. The camera gain and exposure times have to be adapted empirically for your laser and camera specifications to avoid signal saturation. The camera gain may also need to be increased for low abundance proteins.
   b. Take photos every 6.5 min for 6.5 h, or adjust the time intervals to the speed of your microscope (for example every 8 min for 8 hrs) to obtain 61 photos for each position.

Note: The microscope is equipped with the Nikon Perfect Focus System (PFS), which maintains and adjusts focus during the entire time course.

Image processing

⊙ Timing: 3–4 days

This step describes the Image processing steps before quantitative analysis.
Day 4

21. Process images with ImageJ using a custom made macro (enclosed in the supplementary file: Data S1_image-process-macro-forBudJ_related_to_step21.ijm) that:
   a. merges the 5 Z stack projections into one image and keeps the maximum intensity pixels from each stack.
   b. merges the LED Trans, 488 nm and 561 nm channels and displays them by “color”.
   c. concatenates all 61 time points into one file.

You can find an example of the image output after using the macro in the supplementary file Data S2_H4_Pos_11_input_images_for_BudJ_related_to_step21.tif. This tiff file is used as input for BudJ analysis in step 22. We have added the following examples of movies for the red, green and LED trans channels that were generated after the image processing step: Methods video S1:H4dendra2_red, related to step 21, Methods video S2: H4dendra2 green, related_to_step 21 and Methods video S3: H4dendra2_LED-Trans, related step 21.

22. Images are further analyzed with the BudJ (Ferrezuelo et al., 2012) plugin as shown in Figure 5. The link to download BudJ is provided in the Key resources table.

   Note: Copy/paste the downloaded BudJ files into the plugin folder of ImageJ. You also need to download and copy/paste a jama.jar file (any version) into the jars folder of ImageJ.

EXPECTED OUTCOMES

Depending on the number of positions and the number of wells on the slide as well as the initial cell density of the culture, one should expect to get “movies” of 100–300 mother cells that have produced at least two daughters during the time-course. Photo-conversion should be at least 50% efficient in most cells. Red fluorescence should decay over time and green fluorescence should increase. Examples of movies for the red, green and LED-trans channels for one H4-dendra2 mother cell are shown in Methods videos S1, S2, and S3, respectively.

QUANTIFICATION AND STATISTICAL ANALYSIS

We use custom made Perl scripts to analyze the data generated by BudJ. The scripts are provided in the Data S3_related_to_Quantification_and_Statistical_Analysis.zip package in Supplementary Files. They are executed with the TrIPPed.bat program in Windows 10 (you need to read the Readme before running TrIPPed.txt file enclosed in the package before running the program). Table 1 shows the raw data generated by BudJ for one H4-dendra2 mother cell (cell 1 in the table) and its two daughters (cells 2 and 3 in the table). Time 0 is the first time point after photo-conversion. Images were taken every 8.5 minutes. Fluorescence Intensities for each mother cell and its first and second daughter are first analyzed separately and then compiled for all cells. Red (FL1 in Table 1) and green (FL2 in Table 1) fluorescence intensities represent photo-converted and newly synthesized proteins, respectively. Both intensities at each time point are first subtracted from background fluorescence. Background fluorescence for the red channel is determined from the average signal intensity after the signal reached a plateau and does not decrease any more. First, we find the minimal signal intensity over the whole time course (Rmin), which occurs 476 min after photo-conversion for the example in Figure 6 and Table 1. Then we determine the average (Ravgmin) and standard deviation (STD_devavgmin) of the red signal for all time points after the time point when the signal has reached minimum (744 in Figure 6 and Table 1). We consider that the red signal has reached background levels when it falls below Rmin+4* STD_devavgmin (894 in Figure 6). In the example in Figure 6 the red signal falls below the background threshold 221 min after photo-conversion. We then calculate the average intensity of the red signal from that point up to the end of the time course and subtract that value (857 in Figure 6) from intensities from all time points. The background for green fluorescence is determined in the same way except that the average green signal intensity below the
Figure 5. BudJ analysis

(A and B) Start BudJ after opening the image and choose the parameters. The parameters shown in (B) are optimal for our experimental conditions, they should be adjusted for cell size and image quality. Note that we use a scale factor of 1 micron/pixel instead of the actual scale factor for our microscopy set-up of 0.267 microns/pixel to speed up BudJ analysis (using the real scale factor dramatically slows down processing speed). Consequently, the max cell radius parameter of 20 «microns» actually corresponds to 5.34 microns (20*0.267), which is within the radius range of an average sized mother cell. Note also that if you keep the artificial scale factor of 1 and need to use the cell dimensions measured by BudJ, you will have to scale them according to the real scale factor (rsf) calculated for your specific microscope set up (rsf= ([camera pixel size(mm)] * [binning / [objective magnification]]) / [lens factor]; for our microscope rsf=(16mm*[1/60])/1=0.267).

(C and D) Choose one cell. This will be the Mother cell that BudJ will track in all 61 frames (left panel: 561 nm channel, right panel: LED Trans channel of the same field and time point). The red squares indicate the zoomed area shown in (E).

(E) Top row: Identify the time points (magenta arrow) when the fluorescent signal has been fully transferred from the mother (left panel, red arrow) to daughter 1 (middle panel, red arrow) and daughter 2 (right panel, red arrow). Middle and bottom rows: Click on the mother at time 1 (this is cell 1), the first daughter at time 11 (this is cell 2) and the second daughter at time 22 (cell 3) (white arrows). BudJ will now track all three cells until the last frame and record the red and green fluorescence intensities in each cell in every frame.

(F) Output table with fluorescence data. This file can be saved in the .csv format.
Table 1. Example of raw data obtained with BudJ: H4-dendra2 analysis in one mother cell and its two daughters

| Cell (1:Mother: 2:Daughter 1; 3:Daughter 2) | Time (min) | x (coordinate for the position of the mother cell in the image) | y (coordinate of the position of the mother cell in the image) | FL1 (red) FL mean | FL2 (green) FL mean |
|---------------------------------------------|------------|-------------------------------------------------|-------------------------------------------------|------------------|-------------------|
| 1                                           | 0          | 410                                             | 351                                             | 1533.44          | 1629.34           |
| 1                                           | 8.5        | 412                                             | 350                                             | 1792.41          | 1770.34           |
| 1                                           | 17         | 412                                             | 349                                             | 1884.97          | 1869.24           |
| 1                                           | 25.5       | 412                                             | 349                                             | 1794.22          | 1921.31           |
| 1                                           | 34         | 412                                             | 349                                             | 1486.39          | 1898.46           |
| 1                                           | 42.5       | 411                                             | 350                                             | 1764.89          | 2057.41           |
| 1                                           | 51         | 411                                             | 350                                             | 1725.92          | 2084.9            |
| 1                                           | 59.5       | 411                                             | 351                                             | 1747.33          | 2285.18           |
| 1                                           | 68         | 410                                             | 352                                             | 1812.38          | 2144.07           |
| 1                                           | 76.5       | 411                                             | 350                                             | 1849.68          | 2666.55           |
| 1                                           | 85         | 410                                             | 350                                             | 1483.59          | 2613.76           |
| 1                                           | 93.5       | 411                                             | 351                                             | 1240.08          | 2653.08           |
| 1                                           | 102        | 411                                             | 349                                             | 1478.96          | 2985.18           |
| 1                                           | 110.5      | 409                                             | 353                                             | 1480.65          | 3118.68           |
| 1                                           | 119        | 409                                             | 354                                             | 1249.9           | 2877.13           |
| 1                                           | 127.5      | 408                                             | 354                                             | 1270.33          | 2917.24           |
| 1                                           | 136        | 407                                             | 355                                             | 1209.71          | 3153.21           |
| 1                                           | 144.5      | 407                                             | 356                                             | 1321.63          | 3620.65           |
| 1                                           | 153        | 406                                             | 355                                             | 1272.86          | 3528.97           |
| 1                                           | 161.5      | 405                                             | 355                                             | 1291.55          | 3876.69           |
| 1                                           | 170        | 405                                             | 354                                             | 1287.26          | 4220.36           |
| 1                                           | 178.5      | 405                                             | 355                                             | 1097.43          | 4023.17           |
| 1                                           | 187        | 404                                             | 354                                             | 1170.96          | 4162.64           |
| 1                                           | 195.5      | 404                                             | 353                                             | 1149.75          | 4341.88           |
| 1                                           | 204        | 404                                             | 354                                             | 1006.25          | 4515              |
| 1                                           | 212.5      | 405                                             | 352                                             | 1008             | 4179.66           |
| 1                                           | 221        | 404                                             | 350                                             | 862.14           | 3654.02           |
| 1                                           | 229.5      | 404                                             | 350                                             | 849.08           | 3774.66           |
| 1                                           | 238        | 404                                             | 350                                             | 961.94           | 4060.31           |
| 1                                           | 246.5      | 405                                             | 351                                             | 997.61           | 4344.01           |
| 1                                           | 255        | 405                                             | 352                                             | 1013.31          | 4797.27           |
| 1                                           | 263.5      | 404                                             | 353                                             | 1005.72          | 4967.07           |
| 1                                           | 272        | 403                                             | 354                                             | 937.04           | 5069.29           |
| 1                                           | 280.5      | 404                                             | 354                                             | 858.8            | 4944.16           |
| 1                                           | 289        | 403                                             | 357                                             | 855.05           | 4812.66           |
| 1                                           | 297.5      | 400                                             | 359                                             | 833.01           | 4359.86           |
| 1                                           | 306        | 401                                             | 359                                             | 802.58           | 3992.23           |
| 1                                           | 314.5      | 402                                             | 358                                             | 865.53           | 4178.83           |
| 1                                           | 323        | 401                                             | 359                                             | 851.35           | 4275.52           |
| 1                                           | 331.5      | 400                                             | 360                                             | 869              | 4441.27           |
| 1                                           | 340        | 400                                             | 359                                             | 872.05           | 4636.2            |
| 1                                           | 348.5      | 399                                             | 360                                             | 906.2            | 4353.69           |
| 1                                           | 357        | 400                                             | 357                                             | 875.9            | 4888.38           |
| 1                                           | 365.5      | 400                                             | 358                                             | 912.68           | 4925.36           |
| 1                                           | 374        | 400                                             | 358                                             | 882.66           | 4877.2            |
| 1                                           | 382.5      | 400                                             | 359                                             | 872.4            | 5123.58           |
| 1                                           | 391        | 399                                             | 359                                             | 801.34           | 3808.59           |
| 1                                           | 399.5      | 399                                             | 361                                             | 827.03           | 3557.17           |
| 1                                           | 408        | 398                                             | 359                                             | 767.07           | 3764.95           |
| 1                                           | 416.5      | 399                                             | 358                                             | 846.08           | 4296.37           |
| 1                                           | 425        | 397                                             | 358                                             | 819.05           | 3965.25           |
| 1                                           | 433.5      | 398                                             | 357                                             | 852.01           | 4412.61           |
| 1                                           | 442        | 397                                             | 358                                             | 826.93           | 5022.78           |

(Continued on next page)
Table 1. Continued

| Cell (1:Mother; 2:Daughter 1; 3:Daughter 2) | Time (min) | x (coordinate for the position of the mother cell in the image) | y (coordinate of the position of the mother cell in the image) | FL1 (red) FL mean | FL2 (green) FL mean |
|---|---|---|---|---|---|
| 1 | 450.5 | 397 | 359 | 799.09 | 4863.81 |
| 1 | 459 | 397 | 360 | 792.62 | 4562.57 |
| 1 | 467.5 | 396 | 360 | 750.91 | 4426.73 |
| 1 | 476 | 396 | 362 | 744.19 | 3992.52 |
| 1 | 484.5 | 395 | 363 | 792.94 | 3605.57 |
| 1 | 493 | 393 | 360 | 830.92 | 4222.53 |
| 1 | 501.5 | 390 | 363 | 814.19 | 4788.4 |
| 1 | 510 | 391 | 364 | 828.73 | 4257.32 |
| 2 | 93.5 | 412 | 333 | 861.56 | 1861.83 |
| 2 | 102 | 413 | 335 | 934.71 | 1826.24 |
| 2 | 110.5 | 413 | 336 | 1106.14 | 3736.3 |
| 2 | 119 | 413 | 338 | 982.79 | 3946.5 |
| 2 | 127.5 | 414 | 336 | 1334.18 | 3189.7 |
| 2 | 136 | 413 | 336 | 1192.03 | 3194.91 |
| 2 | 144.5 | 412 | 337 | 1239.05 | 3707.7 |
| 2 | 153 | 412 | 336 | 1320.72 | 3650.16 |
| 2 | 156.5 | 411 | 338 | 1229.74 | 3923.89 |
| 2 | 170 | 411 | 338 | 1106.14 | 3736.3 |
| 2 | 178.5 | 411 | 338 | 982.79 | 3946.5 |
| 2 | 187 | 411 | 336 | 1096.34 | 4191.24 |
| 2 | 195.5 | 411 | 336 | 1067.78 | 4462.67 |
| 2 | 204 | 411 | 338 | 1007.47 | 4635.41 |
| 2 | 212.5 | 411 | 336 | 1114.27 | 5198.43 |
| 2 | 221 | 411 | 337 | 1025.88 | 5371.14 |
| 2 | 229.5 | 411 | 336 | 907.69 | 4688.48 |
| 2 | 238 | 411 | 337 | 973.37 | 4231.01 |
| 2 | 246.5 | 411 | 336 | 890.56 | 4218.92 |
| 2 | 255 | 412 | 336 | 923.01 | 4327 |
| 2 | 263.5 | 412 | 337 | 985.43 | 5160.08 |
| 2 | 272 | 410 | 336 | 864.08 | 4802.84 |
| 2 | 280.5 | 410 | 336 | 831.84 | 4996.08 |
| 2 | 289 | 411 | 336 | 831.59 | 4399.26 |
| 2 | 297.5 | 411 | 336 | 883.24 | 5642.43 |
| 2 | 306 | 407 | 342 | 850.1 | 5412.11 |
| 2 | 314.5 | 409 | 342 | 867.92 | 5311.6 |
| 2 | 323 | 409 | 343 | 909.43 | 5856.87 |
| 2 | 331.5 | 409 | 339 | 877.68 | 5369 |
| 2 | 340 | 409 | 334 | 911.14 | 5763.52 |
| 2 | 348.5 | 410 | 332 | 896.16 | 5827.69 |
| 2 | 357 | 409 | 331 | 867.91 | 5744.01 |
| 2 | 365.5 | 410 | 333 | 859.1 | 5411.44 |
| 2 | 374 | 412 | 333 | 861.5 | 5640.01 |
| 2 | 382.5 | 411 | 335 | 849.33 | 5668.69 |
| 2 | 391 | 412 | 335 | 841.25 | 6573.79 |
| 2 | 399.5 | 413 | 334 | 832.83 | 6318.33 |
| 2 | 408 | 412 | 333 | 755.48 | 4745.2 |
| 2 | 416.5 | 413 | 332 | 792.35 | 5106.1 |
| 2 | 425 | 415 | 330 | 819.12 | 5416.82 |
| 2 | 433.5 | 414 | 331 | 828.25 | 5495.94 |
| 2 | 442 | 415 | 330 | 787.73 | 5470.04 |
| 2 | 450.5 | 416 | 331 | 762.14 | 5117.8 |
| 2 | 459 | 415 | 328 | 787.83 | 5697.42 |
| 2 | 467.5 | 416 | 327 | 756.5 | 5640.93 |

(Continued on next page)
minimum ($G_{avg(min)}$) is determined with time points before the time point with the minimal green signal. Since that time point is most often at $t=0$ min, the background level for the green signal ends up being the green signal intensity at time 0 for most cells, i.e., 1692 for the example in Table 1 (Figure 6).

The background corrected intensities are then used to determine: 1. Average cell cycle length; 2. protein decay and synthesis rates; 3. the fraction of “old” proteins retained in the mother after the budding of the first daughter.

1. Average cell cycle length

| Cell (1:Mother: 2:Daughter 1; 3:Daughter 2) | Time (min) | $x$ (coordinate for the position of the mother cell in the image) | $y$ (coordinate of the position of the mother cell in the image) | FL1 (red) FL mean | FL2 (green) FL mean |
|--------------------------------------------|------------|---------------------------------------------------------------|---------------------------------------------------------------|-------------------|-------------------|
| 2                                          | 476        | 416                                                          | 328                                                          | 763.64            | 6310.01           |
| 2                                          | 484.5      | 416                                                          | 327                                                          | 806.55            | 5981.2            |
| 2                                          | 493        | 414                                                          | 324                                                          | 837.99            | 5917.35           |
| 2                                          | 501.5      | 415                                                          | 326                                                          | 813.32            | 5854.22           |
| 2                                          | 510        | 415                                                          | 325                                                          | 830.41            | 6420.86           |
| 3                                          | 229.5      | 388                                                          | 345                                                          | 887.51            | 4891.28           |
| 3                                          | 238        | 389                                                          | 344                                                          | 1022.28           | 5516.39           |
| 3                                          | 246.5      | 390                                                          | 345                                                          | 1045.35           | 6525.26           |
| 3                                          | 255        | 390                                                          | 348                                                          | 1041.64           | 5911.05           |
| 3                                          | 263.5      | 389                                                          | 349                                                          | 982.1             | 5646.12           |
| 3                                          | 272        | 388                                                          | 349                                                          | 880.26            | 5480.79           |
| 3                                          | 280.5      | 388                                                          | 347                                                          | 841.88            | 5783.37           |
| 3                                          | 289        | 390                                                          | 347                                                          | 921.59            | 6749.74           |
| 3                                          | 297.5      | 388                                                          | 346                                                          | 878.91            | 5986.21           |
| 3                                          | 306        | 387                                                          | 346                                                          | 914.89            | 6325.26           |
| 3                                          | 314.5      | 385                                                          | 346                                                          | 907.65            | 6890.88           |
| 3                                          | 323        | 384                                                          | 347                                                          | 912.39            | 6541.34           |
| 3                                          | 331.5      | 385                                                          | 348                                                          | 858.59            | 5498.26           |
| 3                                          | 340        | 387                                                          | 347                                                          | 845.33            | 5356.36           |
| 3                                          | 348.5      | 387                                                          | 347                                                          | 850.4             | 4948.98           |
| 3                                          | 357        | 386                                                          | 346                                                          | 850.03            | 5098.73           |
| 3                                          | 365.5      | 386                                                          | 348                                                          | 859.02            | 5354.93           |
| 3                                          | 374        | 386                                                          | 347                                                          | 864.92            | 5522.11           |
| 3                                          | 382.5      | 386                                                          | 348                                                          | 902.52            | 6401.6            |
| 3                                          | 391        | 385                                                          | 349                                                          | 893.81            | 6580.77           |
| 3                                          | 399.5      | 382                                                          | 350                                                          | 870.73            | 5807.14           |
| 3                                          | 408        | 381                                                          | 348                                                          | 794.62            | 5577.71           |
| 3                                          | 416.5      | 380                                                          | 347                                                          | 870.32            | 5822.2            |
| 3                                          | 425        | 378                                                          | 345                                                          | 880.48            | 5607.99           |
| 3                                          | 433.5      | 381                                                          | 345                                                          | 906.38            | 5830.35           |
| 3                                          | 442        | 380                                                          | 346                                                          | 849.79            | 5688.29           |
| 3                                          | 450.5      | 378                                                          | 349                                                          | 792.26            | 4100.27           |
| 3                                          | 459        | 378                                                          | 350                                                          | 832.92            | 3459.77           |
| 3                                          | 467.5      | 379                                                          | 350                                                          | 804.48            | 4486.64           |
| 3                                          | 476        | 381                                                          | 353                                                          | 807.53            | 4858.49           |
| 3                                          | 484.5      | 380                                                          | 353                                                          | 895.81            | 6067.11           |
| 3                                          | 493        | 378                                                          | 351                                                          | 932.61            | 5849.29           |
| 3                                          | 501.5      | 379                                                          | 353                                                          | 928.46            | 6925.24           |
| 3                                          | 510        | 377                                                          | 354                                                          | 954.92            | 5748.45           |
The length of the cell cycle for each division is equal to: $t(C_n) - t(C_{n-1})$, where $t(C_n)$ is the time of appearance of cell $n$ and $t(C_{n-1})$ is the time of appearance of cell $n-1$. Consequently, in the example in Table 1 the generation time for the first and second budding events are 93.5 min (cell 2: 93.5 min (cell 2) - 0 min (cell 1)) and 136 min (cell 3: 229.5 min (cell 3) - 93.5 min (cell 2)), respectively (Figure 6). The generation times for all budding events of all the mother cells in a given experiment are then averaged to estimate the mean cell cycle length for the whole cell population ($T_{gen avg} \sim 130$ min for the H4-dendra2 strain grown on synthetic media with dextrose).

2. Protein decay and synthesis rates

Protein decay rates are determined from the decay of red fluorescence in the mother cell over the first cell cycle after photo conversion (equal to $L_1-2^*Dt$, where $L_1$ is the length of the first cycle from 1. and $Dt$ is the interval between time points, i.e., 8.5 min for the example in Table 1), i.e., before the first budding event. All time points before the first budding event for each mother cell are normalized to the cell cycle length before the first budding event, and their corresponding red fluorescence intensities are normalized to the average fluorescence intensity over the first cell cycle for each mother cell. The double normalization of time points and fluorescence intensities allows us to group all decay curves from all mother cells into one plot and determine the average decay rate per generation ($D$) from the linear fit of the decay curve (Figure 7). The procedure is similar for the calculation of synthesis rates ($S$), which are determined from the fold increase in green fluorescence over one cell generation. (Figure 7). The decay and synthesis rates expressed in fold change per cell generation are converted to fold change per minute by dividing with the average cell cycle length determined in 1.

3. The fraction of “old” proteins retained in the mother after the budding of the first daughter

The fraction of old protein retained in the mother cell after mitosis ($F_M$) is expressed with the $M/(M+D)$ ratio. $M$ is the average red signal in the mother cell between the first and second budding events and $D$ is the average red signal in the first daughter cell (the first budding event) during the same time period (from the first to the second budding of the mother cell). $F_M$ is determined for each mother cell (Figure 8A) and the mean and median fraction of old protein retained in the mother is determined from the distribution of all $F_M$ for all mother/daughter pairs (Figure 8B).

LIMITATIONS

It is difficult to follow a mother cell for more than two budding events as cells become very dense and start to overlap. Note that the number of generations for which we can follow the red signal is limited.
by the POI half-life and inheritance pattern and since most proteins we have tested have a half-life of one generation, the red signal was typically lost after two generations.

Culture conditions cannot be changed after cells are mounted onto the microscope slide. A microfluidics set up should be combined with the Dendra2 system if reagents need to be added or removed during imaging.

There is a trade-off between the need for higher time resolution and the need for high numbers of analyzed cells. If the experiment requires shorter time intervals between frames and a shorter total photo-conversion time, one needs to reduce the number of positions and/or wells for image acquisition. In that case the experiment will need to be repeated more times to get a sufficient number of cells for statistical analysis.

Image analysis with BudJ is time-consuming and automatic cell and lineage tracking software should be developed to increase the throughput capacity of the method and be able to screen large numbers of cells and strains.

**TROUBLESHOOTING**

**Problem 1**
Cells didn’t grow in the initial culture (steps 1–4).

**Potential solution**
Check the pH of your medium. The pH of synthetic media sometimes needs to be adjusted and can be sensitive to the type of water used. You may have to use deionized water instead of double distilled water. pH should be between 4 and 6.

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**Figure 7. Calculation of H4 synthesis and decay rates**

(A) H4-dendra2 red and green fluorescence intensities over the first cell cycle after photo-conversion for the mother cell (cell 1) from Table 1.

(B) Red (left) and Green (right) fluorescence decay in one generation for 156 and 115 cells, respectively.
Problem 2

Cells grew in the initial culture but did not grow or grew slowly during image acquisition (step 20.)

Potential solution

The composition and/or the pH of the agarose gel medium covering the cells may be “off” because of water evaporation during gel preparation (see step 6). Cells may also have been “shocked” during centrifugation before they were mounted on the slide, which made them enter into lag phase. Be sure to centrifuge them at low g forces (not more than 3500Xg) and pre-heat the centrifuge to 30°C (see step 10.). Finally, if a change in medium composition is required between the liquid culture and the agarose gel such as a carbon source shift, cells will enter a lag phase and take some time to restart dividing. In that case, they should be incubated in the new medium for ~1hr before they are mounted on the slide.

Problem 3

The red signal is too low (step 20.).

Potential solution

1. If the cellular abundance of a protein of interest is low (in which case the green signal before photo-conversion should also be weak), a double or triple Dendra2 tag instead of the single tag can be used to boost the signal (see step 1 in “before you begin”). In our experience the double tag will probably be needed for proteins with 500 to 1000 copies per cell and the triple tag may help with proteins with less than 500 copies/cell. We do not recommend a systematic use of a double or triple tag for all proteins if the single tag produces a good signal, as bulky tags are more likely to destabilize the protein or interfere with its function. Note that a weak signal is any signal that is below the background threshold as defined in Figure 6. The signal can also be amplified by adjusting the sensitivity of the camera. We use a camera gain of 300 for abundant proteins like histones and a gain of 1000 for low abundance proteins like the linker histone H1, but this parameter will have to be optimized for your camera set-up (see step 20a).

2. If photo-conversion efficiency is too low (in which case the green signal is strong), the strength of the LED lamp, the exposure time or the type of neutral density filter have to be adjusted to increase photo-conversion efficiency (see step 19.).
Problem 4
Cell density on the slide is too high or too low (step 10.).

Potential solution
You need to adjust the OD_{600} of the concentrated cell suspension. We use an OD_{600} of 2.2, which is optimal for our experimental conditions. The optimal cell density is 6 to 25 cells per position. If you get more or less, you should dilute or concentrate your cells to a lower or higher OD_{600} than 2.2, respectively.

Problem 5
BudJ does not delimit cells correctly or does not track cells correctly from one frame to the next (step 22).

Potential solution
The parameters for cell size (max. cell radius) and cell boundary (max window for cell edge and max gray change at cell edge) shown in Figure S5B are not correct and they have to be readjusted empirically to better match the cellular characteristics of your particular strain. The “max gray change at cell edge” parameter is sensitive to the quality of the image. We typically use 3% for good quality images and 5% to 6% for “grainier” images.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marta Radman-Livaja (marta.radman-livaja@igmm.cnrs.fr).

Materials availability
All unique/stable reagents generated in this study are available from the lead contact without restriction.

Data and code availability
All microscopy images, Perl and R scripts are available upon request from the lead contact. We additionally provided the Perl scripts in the forTriPP.zip package and one raw image file in Supplementary Files.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100557.

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AUTHOR CONTRIBUTIONS
M.A. optimized and designed the protocol and wrote the manuscript. P.V. constructed the pDendra-NatX plasmid and the H4-dendra2 yeast strain and did the initial protocol development. M.R.-L. conceived TriPP, designed the protocol, developed the analysis pipeline after the BudJ step, and wrote and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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