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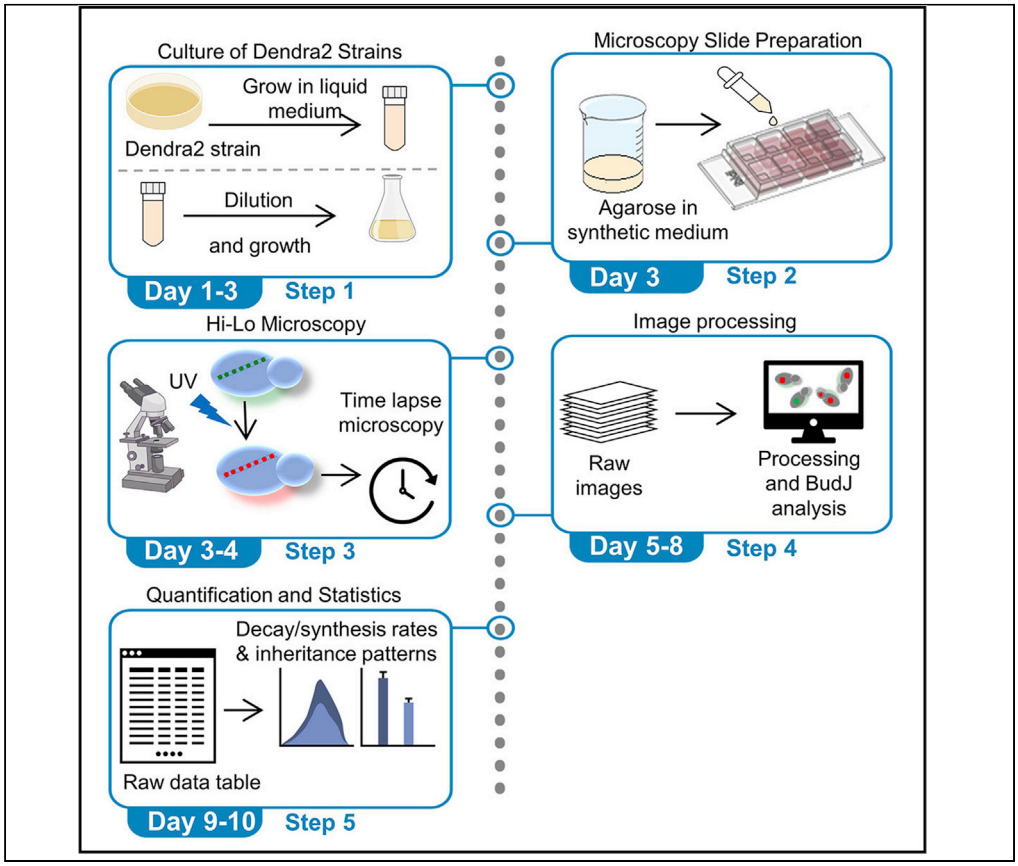
Protocol for tracking the inheritance patterns of proteins in live *Saccharomyces cerevisiae* cells using a photo-convertible fluorescent protein



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.

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Highlights

TriPP is a live-cell imaging technique based on the photo-convertible protein Dendra2

TriPP reveals segregation patterns of maternal proteins after mitosis

TriPP produces protein decay and synthesis rates from single-cell observations

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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 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.

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

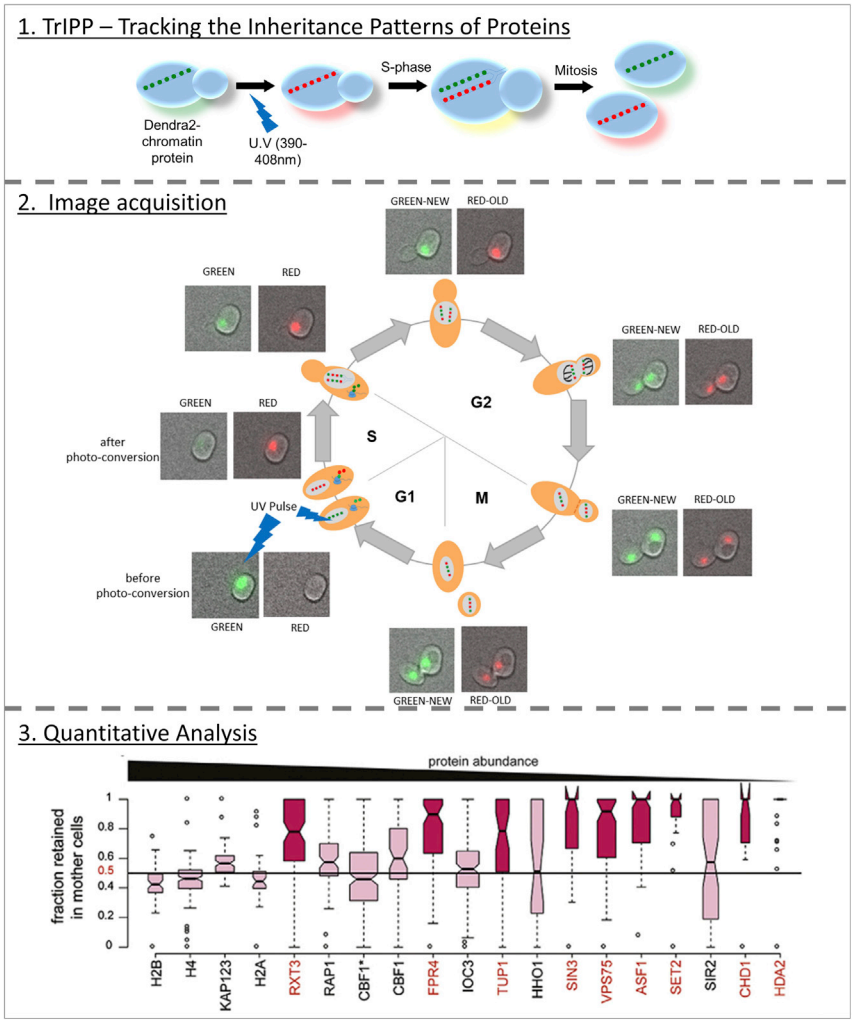

Figure 1. TrIPP principle and expected results

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.

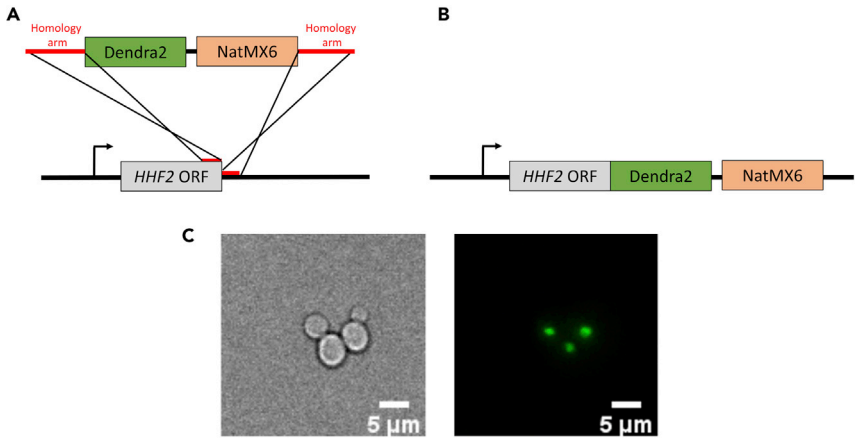

Figure 2. Construction of the H4-Dendra2 strain (HHF2 locus)
(A) Diagram of Dendra2 insertion at the C-terminus of HHF2.
(B) Diagram of final construct after homologous recombination.
(C) Images of H4-Dendra2 taken with a TIRF microscope in Hi-Lo mode. Visible light (left) and green channel before photo-conversion (right).

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. 1× 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	A5131
Histidine	Sigma	H8125
Isoleucine	Sigma	I2752
Leucine	Sigma	L8000
Lysine	Sigma	L5626
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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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agarose Standard	Eurobio	n°GEPAGA07-65
Experimental models: Organisms/strains		
Saccharomyces cerevisiae (*MATa ura3Δ leu2Δ his3Δ met15Δ bar1Δ::HIS5 HHF2-Dendra2::NatMX*, BY4741 background)	Auboiron et al. (2021)	n/a
Oligonucleotides		
HHF2dendranatxF (forward integration primer for the *HHF2* ORF): TTTGAAGAGACAAGGTAGAACCTTATATGGTTTCGGTGGTG GTGGCGGAAACACCCCGGGAATTAACCT	This study	n/a
HHF2dendranatxR (reverse integration primer for *HHF2* ORF): TTTGAAGAGACAAGGTAGAACCTTATACGGTTTCGGTGGTGG TGGCGGAAACACCCCGGGAATTAACCT	This study	n/a
HHF2denVF (forward verification primer for *HHF2* ORF): TTACTTCTTTGGATGTTGTT	This study	n/a
HHF2denVR (reverse verification primer for *HHF2* ORF): ATTCCCAATAGAATGATCGT	This study	n/a
Recombinant DNA		
pDendraNatMX6	This study	n/a
Software and algorithms		
MetaMorph (for the microscope)	Molecular Devices	n/a
ImageJ	Wayne Rasband, NIH, USA	n/a
BudJ plugin. Downloadable here: https://www.researchgate.net/post/Where-I-can-download-the-plugin-BudJ-to-use-in-the-program-ImageJ-for-yeast-volume-analysis	Martí Aldea, Institut de Biologia Molecular de Barcelona	n/a
forTrIPP.zip. Perl package for data analysis after BudJ processing. Available in Supplementary Files.	This study	n/a
Other		
Spectrophotometer Ultrospec 10 Cell Density Meter	Amersham Biosciences	n/a
aPES membrane filter	Thermo Scientific	566-0020
Microscopy slide μ-Slide 8 Well Glass Bottom	ibidi	n°80827

MATERIALS AND EQUIPMENT

- YNB 10×

Reagent	Final concentration	Amount
YNB (Yeast Nitrogen Base) without ammonium sulfate	19 g/L	19 g
Ammonium sulfate	50 g/L	50 g
ddH_2O		Up to 1 L
Total	**10×**	**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 100×

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*)

Continued

Reagent	Final concentration	Amount
Tryptophan	4 g/L	4 g
ddH_2O		Up to 1 L
Total	**100×**	**1 L**

Filter sterilize with 0.2 μm filters. Keep at 4°C protected from light (indefinitely if not contaminated).

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 100×

Reagent	Final concentration	Amount
Adenine	5 g/L	2 g
ddH_2O		Up to 400 mL
Total	**100×**	**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 8×

Reagent	Final concentration	Amount
Tyrosine	0.4 g/L	0.4 g
ddH_2O		Up to 1 L
Total	**8 ×**	**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 50×

Reagent	Final concentration	Amount
Uracil	2.5 g/L	1.25 g
ddH_2O		Up to 500 mL
Total	**50×**	**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% (10×)

Reagent	Final concentration	Amount
Glucose or Galactose	20%	200 g
ddH_2O		Up to 1 L
Total	**10×**	**1 L**

Filter sterilize with 0.2 μm filters in sterile bottle.

- COMPLETE SYNTHETIC MEDIUM 1×

The 1× medium solution is assembled from all the above stock solutions. Note that the final pH should be between 4 and 6 for optimal growth.

Reagent	Final concentration	Amount
YNB 10×	1×	100 mL
Drop-out 100×	1×	10 mL
Adenine 100×	1× (50 mg/L)	10 mL
Tyrosine 8×	1× (50 mg/L)	125 mL
Uracil 50×	1× (50 mg/L)	20 mL
Glucose or Galactose 20% (10×)	1× (2%)	100 mL
ddH_2O		635 mL
Total	1×	1 L

Note: Combine all solutions in a sterilized glass bottle and keep at 23°C–25°C. Handle all I sterile conditions (next to a flame or cell culture hood) to avoid contamination. Make just enough 1× 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 OD_{600} of the culture and inoculate 20 mL of the same synthetic medium with the appropriate volume of the overnight (~16hrs) culture so that the OD_{600} is ~0.3 the next day (see calculations below). Incubate overnight (~16hrs) at 30°C and 220 rpm
   a. OD_{600} = 0.5 corresponds to 10^7 cells/mL, as measured with our spectrophotometer
   b. The inoculation volume of the initial culture needed to obtain a culture with the desired OD_{600} after a defined time interval is calculated with the following formula:

$$\frac{OD_{600}final \times Vfinal}{2^{\frac{t}{g}} \times OD_{600}initial} = Vinitial$$

with:

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

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×*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 the 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×*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.

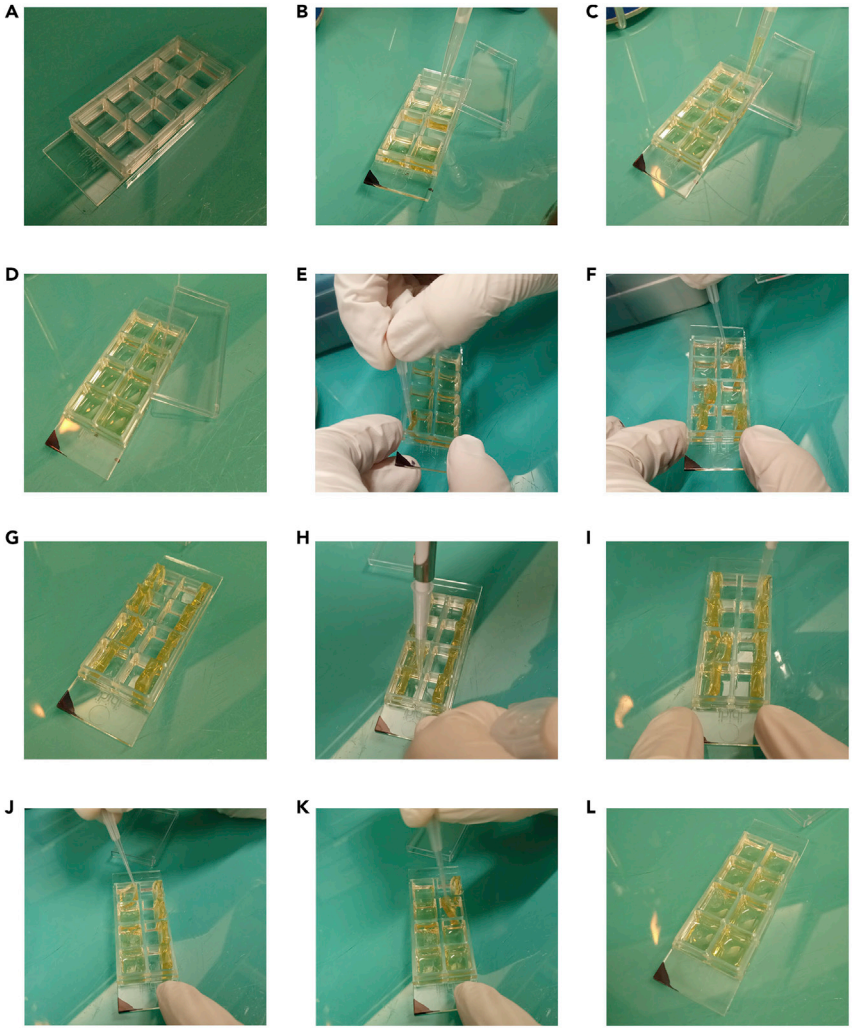

Figure 3. Microscopy slide preparation (steps 6 to 10 of the protocol)

(A–D) Fill all 8 wells with liquid agarose solution (~350μL per well) and let the gel solidify in the wells.

(E–G) Use a sterile 10 μL pipette tip to carefully lift the gels and place them vertically on the side of each well. Wait a few minutes for the bottom of the well to dry.

(H and–I) Put 3 μL of yeast cell suspension into each well and spread the liquid with the pipette tip. Let dry 10 s to 2 min.

(J–L) Carefully put the agarose gel back on top of the cells without trapping bubbles. Cover the slide with its lid.

15. Adjust the laser power and LED lamp (Lumencor system) parameters to:
    a. 488 nm laser: ~360 μW
    b. 561 nm laser: ~300 μW
    c. LED photo-conversion (390 nm): ~2.30 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 μm pixel size)

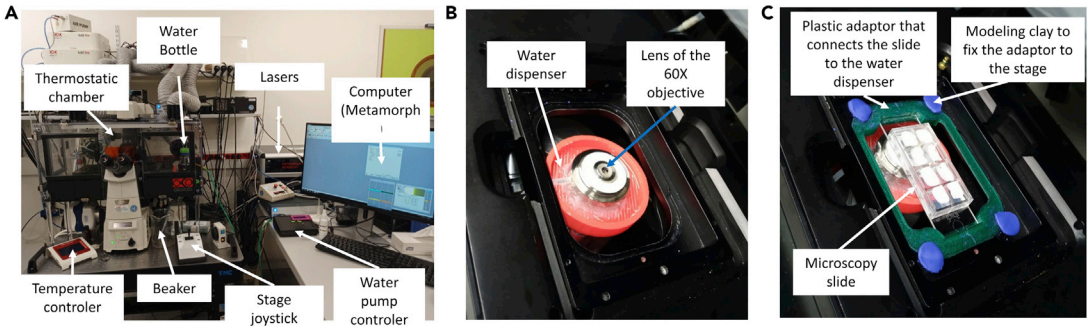

Figure 4. Hi-Lo/TIRF microscope

(A) Microscope set-up.

(B) Custom made water dispenser for the 60× water objective. A bottle of water placed in the thermostatic chamber (30°C) supplies the water for the pump, which continuously dispenses a drop of water onto the lens of the 60× objective. A beaker connected to the water dispenser collects the excess water coming from the pump.

(C) The microscopy slide attached to the water dispenser

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.tiff. 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 (R_{avgmin}) and standard deviation ($STDev_{avgmin}$) 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* $STDev_{avgmin}$ (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

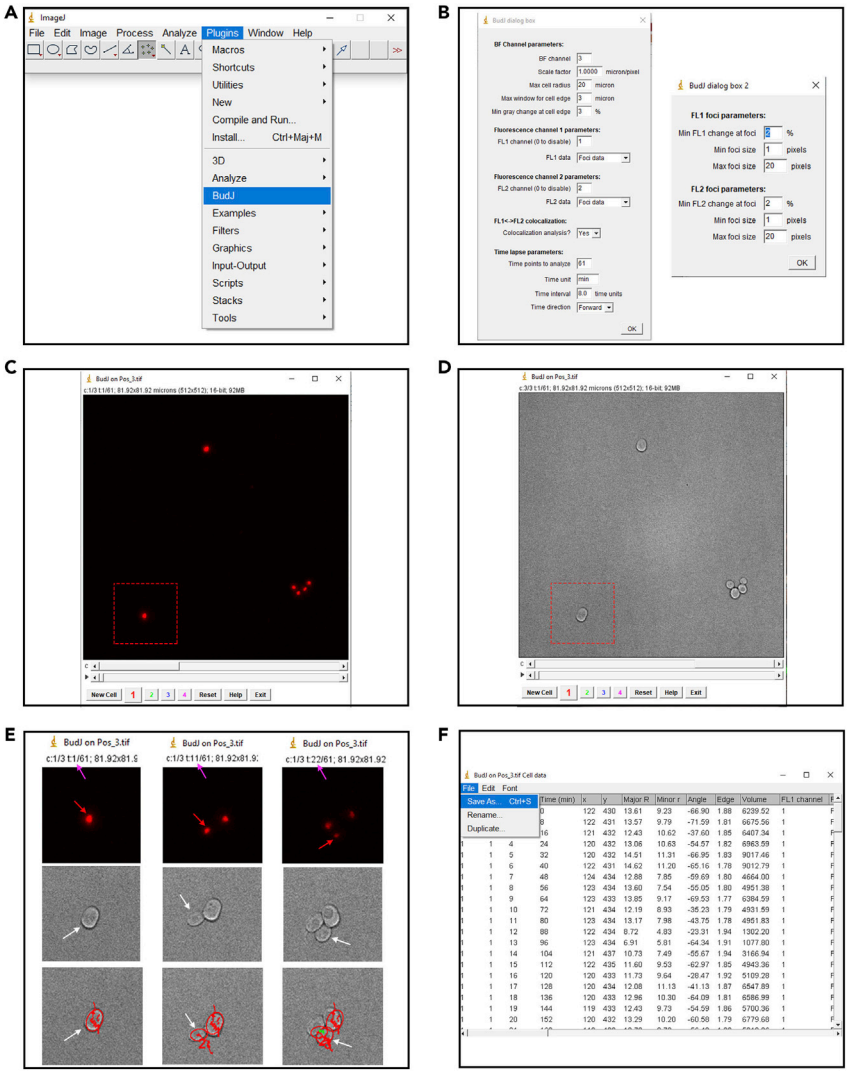

Figure 5. BudJ analysis

(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 0.267microns/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	2414.07
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	771.49	1411.82
2	102	413	335	961.56	1861.83
2	110.5	413	336	934.71	1826.24
2	119	413	338	1206.73	2792.08
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	161.5	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*)

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
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

minimum (G_{avgmin}) 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 Table1 (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

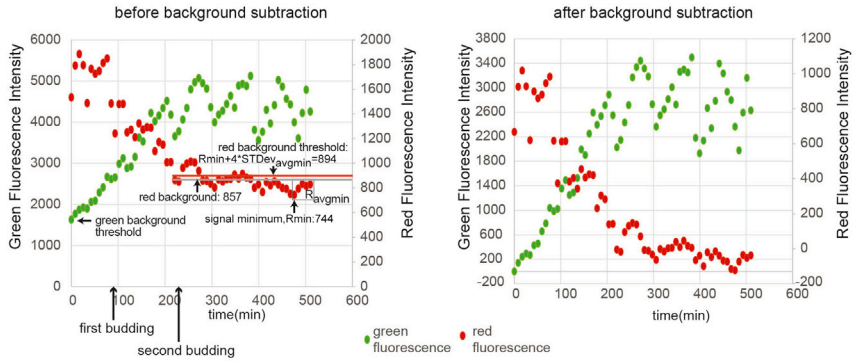

Figure 6. H4-dendra2 red and green fluorescence intensities over the entire time course for the mother cell (cell 1) from Table 1

The length of the cell cycle for each division is equal to: $t(C_n)$-$t(C_{n-1})$, $t(C_n)$ 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_{genavg}~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 L1-2*Δt, where L1 is the length of the first cycle from 1. and Δt 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

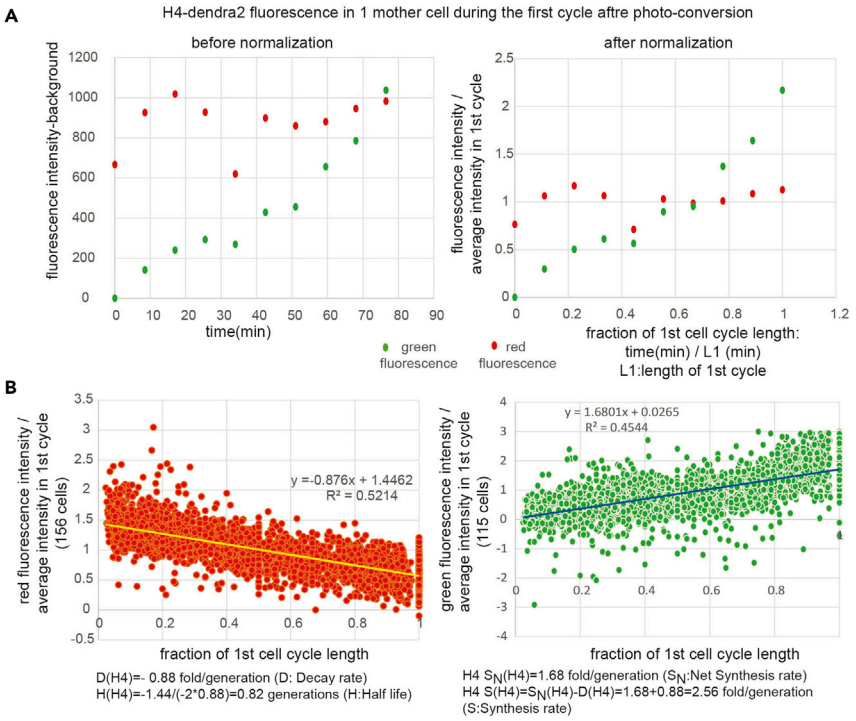

Figure 7. Calculation of H4 synthesis and decay rates

(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.

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.

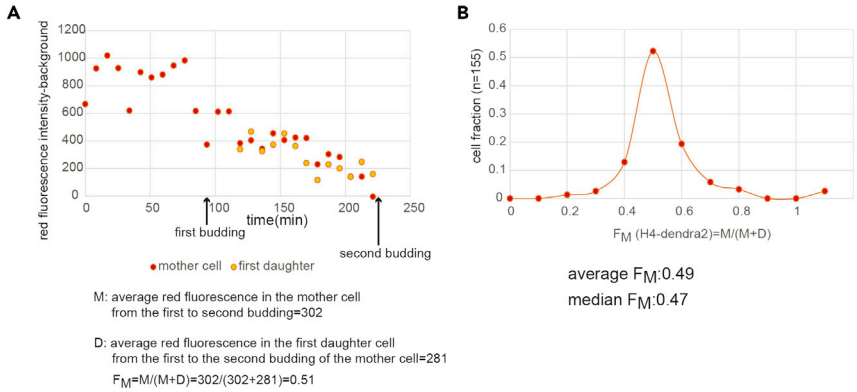

Figure 8. The fraction of "old" H4 retained in the mother after the budding of the first daughter

(A) H4-dendra2 red fluorescence intensities before the second budding of the mother cell (cell 1) from Table 1 in the mother cell (red) and its first daughter (yellow). FM=M/(M+D) is the fraction of the red signal that is retained in the mother cell after the first budding.

(B) Distribution of FM(H4) in 155 mother/daughter pairs.

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 5B 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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