Using bleach-chase to measure protein half-lives in living cells

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Protein removal has a central role in numerous cellular processes. Obtaining systematic measurements of multiple protein removal rates is necessary to understand the principles that govern these processes, but it is currently a major technical challenge. To address this, we developed 'bleach-chase', a noninvasive method for measuring the half-lives of multiple proteins at high temporal resolution in living cells. The method uses a library of annotated human reporter cell clones, each with a unique fluorescently tagged protein expressed from its native chromosomal location. In this protocol, we detail a simple procedure that bleaches the cells and uses time-lapse fluorescence microscopy and automated image analysis to systematically measure the half-life dynamics of multiple proteins. The duration of the protocol is 4–5 d. The method may be applicable to a wide range of fluorescently tagged proteins and cell lines.

INTRODUCTION

Protein removal has a central role in many cellular processes, including the cell cycle, signal transduction and apoptosis[1,2], and abnormalities in protein removal have been implicated in cancer and other human diseases[2–4]. Thus, uncovering the principles that govern protein removal is needed in order to understand how living cells regulate their proteomes. Methods for systematic measurements of protein removal rates in living cells are vital in this effort.

The rate of protein removal in living cells is the sum of two underlying processes: intracellular degradation (e.g., via the proteasome) and dilution due to cell growth, which reduces the protein amount by 50% with every cell division[5,6]. The protein half-life, defined as the time for removal of half of the protein, is $T_{1/2}$ = ln(2) / removal rate.

Methods available to measure protein removal rate

An ideal method for measuring protein removal rates should: (i) allow quantitative simultaneous measurements of multiple proteins, (ii) enable high temporal resolution over many time points, (iii) cause minimal disturbance to the cells, (iv) be simple to apply under various experimental setups, (v) not involve radioactive labeling and (vi) work in living cells.

Pulse-chase, the traditional method, involves radioactive labeling of the protein of interest for a brief period of time ('the pulse'), followed by measurements of the decay in radioactivity over time ('the chase')[7] (**Fig. 1a**). This assay is considered to be the gold standard because it is accurate and causes only minor interference to the cells. However, each protein of interest requires a specific antibody, making it difficult to scale up to multiple proteins. The method is also restricted to measuring a few time points. Protein synthesis inhibitors have also been used to measure protein removal rates[8]. Although they are nonradioactive and easier to apply than pulse-chase, they gradually lead to severe cell interference and are therefore less suitable for measuring the half-lives of long-lived proteins. Global quantification of protein half-lives in mammalian cells that overcomes these limitations is performed by integrating pulse labeling and mass spectrometry[5,9]. However, this approach is less suitable for real-time monitoring of living cells and is restricted to a few time points. Another high-throughput method uses a library of clones, each with a unique pair of fluorescent tags. Coupled with cell sorting and microarrays, the method provides useful information on the proteome's relative turnover rates but does not allow quantitative measurements[10].

To address many of these challenges, we recently developed bleach-chase: a nonradioactive method for accurately measuring protein removal rates of multiple proteins at high temporal resolution while causing minimal interference to the cells[11]. By using bleach-chase, we were able to measure the half-life dynamics of 100 proteins in living human cells, under normal and stress conditions at high temporal resolution (20 min resolution for 24 h)[11]. We used these measurements to study the interplay between protein degradation and dilution under normal conditions and stress. The method builds on a pre-existing library of human non–small cell lung cancer cell line (H1299) in which each clone has a unique fluorescently tagged protein expressed from its endogenous locus[11–14]. The method involves a simple bleaching procedure, coupled with time-lapse microscopy and automated image analysis. An overview of the bleach-chase workflow is presented in **Figure 1b**,**c** and a timing overview is provided in **Table 1** .

Bleach-chase was tested in a series of control experiments[11]. The day-to-day reproducibility measurement error was 0.25 (coefficient of variance (CV)). Bleach-chase compared well with radioactive pulse-chase, the gold standard, showing similar half-lives for tagged proteins (11% median difference, seven proteins). Comparison of the half-lives of tagged proteins with their wild-type counterparts, expressed from the untagged allele in the same cells, showed good agreement as well (16% median difference, six proteins). This is in line with other studies that found good agreement between half-lives and dynamics of tagged and untagged proteins[8,12,15–21]. Bleach-chase did not alter cellular motility, cell cycle duration, viability or morphology, and was found to be roughly insensitive to different intensities of bleaching (CV < 0.2)[11].

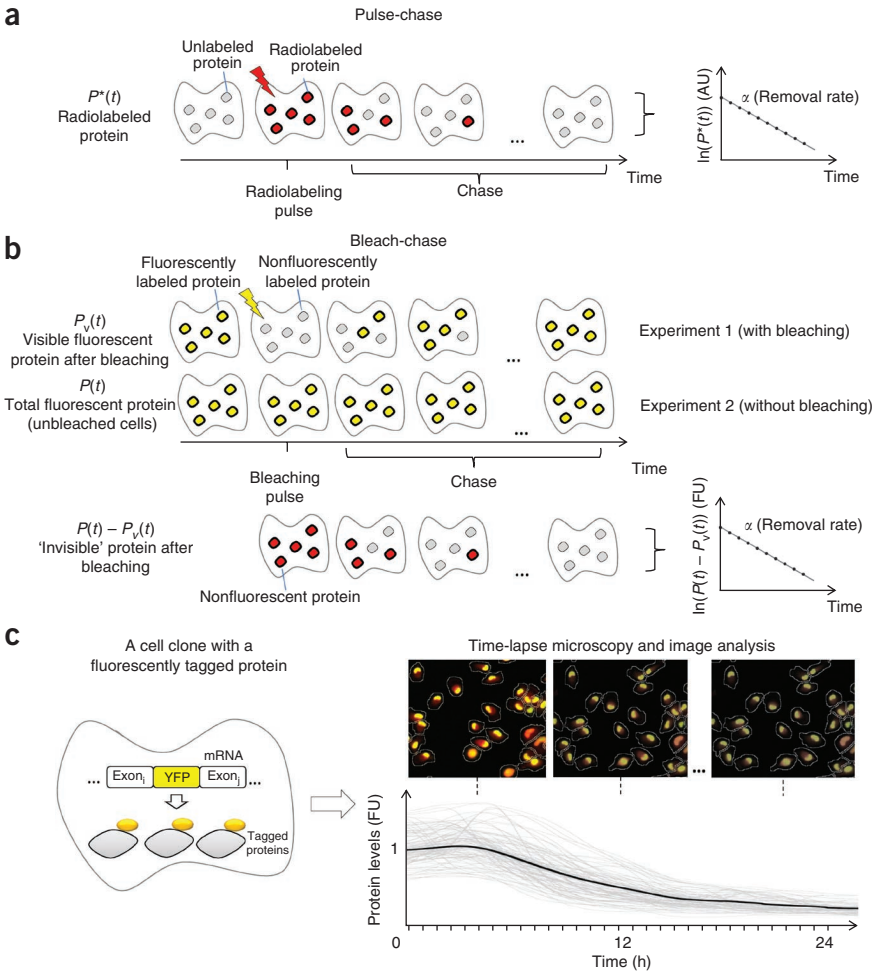


Figure 1 | Workflow of the bleach-chase method and its analogy to pulse-chase. (**a**) Schematics of the pulse-chase workflow: cells are grown for a brief duration in a medium containing radioactively labeled amino acids that are incorporated into the proteome (the 'pulse'). The temporal decay of a radiolabeled protein of interest is measured at consecutive time points (the 'chase'). The protein degradation rate is the slope of decay on a semi-logarithmic plot. The protein half-life, defined as the time for removal of half of the protein, is $T_{1/2} = \ln(2)/\alpha$. (**b**) Schematics of the bleach-chase workflow: the fluorophore of a tagged protein is bleached using a brief pulse of light, irreversibly causing it to become nonfluorescent (experiment 1). This generates two subpopulations of the tagged protein: fluorescent and nonfluorescent (yellow and gray, respectively). After bleaching, the nonfluorescent proteins are no longer produced and therefore decay at a rate that depends solely on protein removal (decay of gray proteins in experiment 1), in a manner analogous to the decay of radiolabeled tagged proteins in the pulse-chase experiment. However, the nonfluorescent proteins are invisible by fluorescence microscopy and cannot be measured directly. To overcome this, we repeat the same experiment without bleaching (experiment 2) and subtract the visible fluorescent signal of the two experiments[11]. (**c**) Bleach-chase was developed and tested on a library of cell clones, each with an endogenously YFP-tagged protein[11]. Fluorescent protein levels were obtained using time-lapse microscopy. Analysis was performed using the PhenoTrack custom software for automated cell segmentation, tracking and quantitative protein measurements. Protein fluorescence and individual cell boundaries in a series of consecutive frames are indicated by yellow regions surrounded by white silhouettes. The population average dynamics (black line) were computed on more than 300 individual cells (gray lines). Fluorescence levels were normalized to the population average at $t = 0$. AU, arbitrary units; FU, fluorescence units.

Although the bleach-chase method was developed and tested on the library of human annotated reporter cell clones (LARC)[11,12], it may be adapted to measure protein half-lives in other cell lines and clone libraries with fluorescently tagged proteins. Here we present a detailed protocol of the bleach-chase method.

Experimental design

The half-lives of fluorescently tagged proteins and their wild-type counterparts are similar. For bleach-chase to work, the half-life of the fluorescently tagged protein should resemble that of the native untagged protein. To assess the degree by which the half-life of a native protein is affected by the insertion of a fluorescent tag, we and others compared the half-lives of tagged with untagged wild-type proteins using pulse-chase and other methods and found good agreement in most of the tested proteins[8,11,15–19]. Notably, one factor that may affect a protein's half-life is its N-terminal amino acid (called the N-terminal rule)[22,23]. In the library used in the present protocol, a yellow fluorescent protein (YFP) tag was inserted into a protein sequence as a new internal exon. Such tagging tends to preserve the N-terminal amino acids of the protein and is therefore less likely to interfere with the degradation signals compared with the N-terminal tagging.

Controls. The following set of controls should be performed when running bleach-chase. This is particularly important when applying bleach-chase to new cell lines on which bleach-chase has not been tested.

- Testing bleaching perturbation: the mild bleaching procedure described in the protocol was designed to minimize cellular perturbation. To examine whether cellular physiology has been altered, one should compare motility, cell cycle duration, morphology and rate of cell death, before and after bleaching. Values should remain roughly unchanged.
- It is important to compare the half-life measurements of the tagged proteins using bleach-chase and pulse-chase on a few selected proteins spanning the entire range of half-lives, and to verify that the two methods yield similar results (an example of such a comparison in human cells is presented in ref. 11).
- When testing bleach-chase on a new library of clones, we recommend that the half-lives of tagged proteins be compared with their non-tagged counterparts in order to verify that the tagging tends to preserve the half-life of the endogenous protein (an example of such a comparison in human cells was presented in ref. 11).

TABLE 1 | Timing.

Day	Procedure	Time	Work involved	Work intensity
1 (Steps 1–8)	Cell thawing	20 min of work, 20–24 h of incubation	Tissue culture and incubating	Low
2 (Steps 9–14)	Plating cells for microscopy experiment on 12-well optical plates	1.5 h per six clones (two wells for each clone)	Tissue culture	Medium
3 (Steps 15–43)	Bleaching and time-lapse movie acquisition	Initial preparation takes roughly 4.5 h. Experiment length depends on the duration of protein half-life measurements (typically ranges between 6 and 48 h)	Tissue culture (changing medium) Determine the appropriate bleaching duration required for each protein Microscope preparation for a movie Choosing FOVs Running the system for several time-lapse rounds (even one is sufficient). Bleaching half of the wells (can take up to 3 h) Adjusting the settings of the time-lapse microscopy after bleaching Time-lapse movie acquisition	Medium-high
4 (Steps 44–46)	Image analysis and protein half-life measurement	About 1 h per video containing 140 frames, when analyzed on an Intel Pentium dual-core E5200, 4G. A typical experiment generates 36 videos, thus requiring 36 h of analysis when running on a single PC. Running analysis in parallel on a computer grid may substantially reduce analysis time	Image analysis and acquisition of protein fluorescence dynamics using the PhenoTrack software. Note: different movies can be run concurrently on multiple PCs or a computer grid to reduce running time Bleach-chase analysis: obtaining half-life measurements based on the protein fluorescence dynamics	Medium

- Bleach-chase half-life measurements should be independent of the degree of bleaching at $t = 0$ (see **Box 1**, equation 4). To verify this, repeat bleach-chase on the same protein using different bleaching exposure times and test whether the measured half-lives remain similar.
- Verify that the growth of the cells under the microscope and in the tissue culture is similar.

Note that the expected day-to-day reproducibility error of the removal rate under the current setting is about 0.4 (CV)[11]. We recommend averaging at least three day-to-day repeats in order to obtain an average reproducibility error of <0.25 (CV).

Applying bleach-chase to other cell lines. The bleach-chase method was developed on the LARC library of human lung cancer cells[11,12]. However, the same principles may be generally applied to other clones and cell lines in which the protein under study is tagged with a fluorophore, either endogenously or exogenously. This is because protein removal using bleach-chase is independent of protein production rate or source. In particular, bleach-chase may potentially be extended to other existing libraries of fluorescently tagged proteins such as the yeast and *Escherichia coli* GFP-tagged protein libraries[8,24,25] and the *Drosophila* protein trap library[26].

Additional applications and uses of bleach-chase. Other potential uses of bleach-chase include:

- *Studying protein removal in different cellular compartments and localizations.* Bleach-chase allows a microscopy-based assay of protein removal and therefore can be used in concert with cellular compartment image segmentation algorithms[11] to measure how a protein is degraded in different localizations (e.g., nucleus versus cytoplasm).
- *Studying protein removal rates as a function of cell cycle stage.* Bleach-chase facilitates the measurements of protein removal rates as a function of cell cycle stage, without the need for chemical or physical synchronization. This can be achieved by applying bleach-chase to a population of unsynchronized cells, followed by *in silico* synchronization[11,14].
- *Estimating protein production rates.* This can be achieved by using the measured removal rate and protein levels and applying equation 3 in **Box 1**.

Box 1 | Bleach-chase equations

(1) $\alpha = \alpha_{deg} + \alpha_{dil}$

(2) $T_{1/2} = \frac{\ln(2)}{\alpha}$

(3) $\frac{dP(t)}{dt} = \beta - \alpha \cdot P(t)$

(4) $\ln(P(t) - P_v(t)) = \ln(P(t_0) - P_v(t_0)) - \alpha \cdot t$

The throughput of bleach-chase. The protocol described herein uses 12-well plates coupled with time-lapse microscopy. Thus, under the current settings, each microscope could generate measurements of up to six proteins simultaneously (two wells per protein for the bleached and unbleached cells). With the current protocol, we were able to measure the removal rate dynamics of 100 proteins for 24 h at 20-min resolution[11]. Such throughput would be difficult to achieve using classical pulse-chase assays. One potential way to increase assay throughput is by recording the measurements using 96-well plates. Another way is to perform bleaching using whole-plate illumination instead of bleaching each field of view (FOV) individually as described in the present protocol.

The temporal resolution limits of bleach-chase. Bleach-chase uses time-lapse microscopy, which can achieve high temporal resolution. However, the temporal resolution is limited by the fluorescent tag folding and maturation time, which can range between a few minutes and 2 h, depending on the fluorophore and the conditions[27–29]. Consequentially, a protein that is constitutively degraded at a rate faster than fluorophore maturation time would not be measurable using bleach-chase.

Determining the bleaching exposure duration for each protein. The bleaching exposure time may vary across different fluorescently tagged proteins. It is preferable to determine the optimal exposure time in advance so that the protein fluorescence levels immediately after the bleaching drop by 30–60% compared with prebleaching levels. Typical bleaching exposure times range between 0.5 and 8 min when using a 120-W mercury fluorescent lamp (other lamps may require different bleaching exposure times). To determine the bleaching exposure time, we recommend using a separate plate with the desired fluorescent clones seeded. Take snapshots of the fluorescence levels after serial exposure times, ranging between 0.5 and 8 min. For each protein, determine the exposure time required in order to reduce fluorescence levels by 30–60% of prebleaching levels (the half-life measurements are roughly independent of the exact amount of bleaching). It is important not to overbleach the cells, as it may cause phototoxicity.

Determining the exposure time for standard fluorescence acquisition. The exposure time required for the YFP and mCherry standard fluorescence acquisition should be determined in advance. To this end, select an exposure time that produces a visible fluorescent signal (strong contrast where the target signal is at least 20% stronger than the background), which is not saturated (i.e., <70% of maximal pixel intensity). The appropriate YFP exposure time may change across different clones and should be adjusted accordingly, whereas that of the mCherry tends to be preserved throughout the LARC library clones.

The YuvControl software for controlling the time-lapse movie acquisition. Our custom YuvControl software allows control over the time-lapse movie workflow. The software also supports autofocusing. This is achieved by performing acquisition of five *z*-planes at 3-μm intervals. The *z*-plane with the highest contrast is obtained and used for the phase and fluorescence (YFP and mCherry) images. The number of *z*-planes and interval size can be adjusted on the YuvControl window in the 'coarse AF' and 'fine AF' fields. The exposure time of the phase images is set at the bottom of the 'YuvControl' window next to AutoFocus (AF). Note that the 'pause' button in the YuvControl window pauses the movie only after the current round is completed. The 'cancel' button stops the movie immediately with no option to continue. Additional time-lapse movie parameters are optional through the YuvControl window and are self-explanatory.

Viewing the movie images at the end of the time-lapse experiment. After the movie acquisition is completed, images can be viewed with the ImagePro program by merging the files and viewing them as a movie. Individual images can be viewed as regular tiff files using a variety of different software programs, such as ImagePro, Irfanview or MATLAB.

Automated time-lapse movie analysis. PhenoTrack is a custom software used in this protocol in order to perform automated time-lapse movie analysis, and it includes background normalization, cell segmentation, cell tracking and automated detection of cellular phenotypes such as mitosis and apoptosis (**Fig. 1c**). Briefly, the cell segmentation relies on the red fluorescent tag, mCherry, common to all clones in the library, which is strongly expressed in the nucleus and less in the cytoplasm. Otsu's approach[30] is used to identify the cutoff between intensity of cell nucleus and cytoplasm; this is followed by seeded watershed segmentation in order to determine cell boundaries[31]. The cell-tracking procedure maps each cell to the appropriate cells in the preceding and following frames. To this end, the trajectory of each cell triplet (in frame $i - 1$, i and $i + 1$) is evaluated and assigned a cost depending on its velocity consistency (large changes in velocity increase the cost) and angle smoothness (large changes in trajectory angle increase the cost). Size and appearance compatibility, as well as mitosis events, are also taken into account. Cell tracking is then resolved by obtaining a nonoverlapping assignment of trajectories that minimizes the cost of all trajectories[32]. The cellular phenotype identification is achieved using a machine learning approach[11]. The PhenoTrack software was written in MATLAB 2007. Other software programs that can extract quantitative traces of protein dynamics from time-lapse movies can be used to achieve similar results.

MATERIALS

REAGENTS

▲ CRITICAL Reagent suppliers described herein were used to develop and test the protocol. Comparable reagents may be acquired from other suppliers.

- Cells expressing a fluorescently tagged protein. In this study, cell clones from the LARC library were used[11] (the bleach-chase method may be generally applied to other cell types expressing a fluorescently tagged protein, upon appropriate adjustments of the protocol).
- Complete transparent growth medium for normal cell growth under the microscope. For the LARC cells, we used RPMI 1640 lacking riboflavin and phenol red (e.g., Bet Haemek, Biological Industries, cat. no. 06-1100-26-1A), supplemented with 10% (vol/vol) fetal calf serum (e.g., Biological Industries, cat. no. 04-001-1A) and 0.05% (vol/vol) penicillin-streptomycin.
- L-Glutamine (e.g., GIBCO, cat. no. 21875)
- Trypsin solution (e.g., Biological Industries, cat. no. 03-053-1)
- DMSO (e.g., Hybri-Max, Sigma, cat. no. D2650)
- Isopropanol for the freezing container ! CAUTION It is flammable. Handle using appropriate safety equipment and measures.
- Liquid nitrogen ! CAUTION It is a harmful solution. Handle it using appropriate safety equipment.
- Ethanol, 70% (vol/vol)
- Fibronectin (1 mg ml^{-1}; e.g., solution from bovine plasma; Sigma, cat. no. F1141)
- Sterile 1× Dulbecco's phosphate-buffered saline (e.g., PBS; Sigma, cat. no. D8537)

EQUIPMENT

- Tissue culture incubator (with 37 °C, 8% CO_2; note: 8% CO_2 for H1299 LARC library cell clones; adjust according to the cells in the study control and humidified air)
- Tissue culture hood (e.g., ADS Laminaire optimal 12, type 2)
- Centrifuge (Eppendorf, cat. no. 5810R)
- Tissue culture dishes (10 cm)
- Tissue culture tubes (15 ml and 50 ml)
- Hemocytometer
- Water bath (37 °C)
- Cryo 1 °C cell freezing containers (Nalgene)
- Optical 12-well glass-bottom plates (e.g., Mat-Tek cultureware, Microwell plates uncoated, part number P12G-0-14-F, lot number TK0289)
- Inverted fluorescence microscopes (Leica, cat. nos. DMIRE2 and/or DMI6000B) or an equivalent
- Plate holder (e.g., 96-well plate holder with top screw removed to enable CO_2 cover placement; Martzhaeuser)
- Microscope-mounted, temperature-controlled 37 °C incubator (37-2 digital and heating unit; PeCon, Leica, cat. no. 15531719)
- Humidity and 8% CO_2 microscope internal control and chambers (e.g., PeCon, cat. no. 0506.000-230, Leica, cat. no. 1152733) ▲ CRITICAL Alternatively, replacement of normal bicarbonate-buffered medium with medium buffered by HEPES will keep cells alive under nonincubator conditions for several hours (up to about 15 h).
- Custom plate enclosure to maintain constant temperature, CO_2 concentration and humidity (internal in the microscope)
- Motorized stage (e.g., Martzhaeuser)
- Automated stage movement control (e.g., Corvus, ITK)
- Electronic shutters (e.g., Uniblitz, cat. no. VMM-D1)
- Fluorescent light source (e.g., Mercury short arc lamp HXP)
- Cooled 12- and 14-bit CCD cameras: QImaging (RETIGA-S&V, Fast 1394, RET-SRV-F-M-12-C); CoolSNAP (Roper Scientific HQ, photometrics); ORCA-ER (Hamamatsu photonics K.K., cat. no. C4742-95-12ERG) or any equivalent ▲ CRITICAL It is important to test the compatibility of the imaging software and the camera before purchase.
- Fluorescent single-channel filter cubes: YFP (500/20-nm excitation, 515-nm dichroic splitter and 535/30-nm emission; Chroma, cat. no. 41028);
- mCherry (575/50-nm excitation, 610-nm dichroic splitter and 640/50-nm emission; Chroma, cat. no. 41043)
- Plan Apochromat air objective (×20, numerical aperture 0.7)
- ImagePro5 Plus or ImagePro 5.1, and Scope Pro software (Media Cybernetics) to operate time-lapse movies on microscopes. ImagePro software integrates time-lapse acquisition, stage movement and software-based autofocus
- YuvControl macro, custom-written program on ImagePro (available from our laboratory)
- Movie calibration macro, custom-written program on ImagePro (available from our laboratory)
- MATLAB 2007 or a newer version (required for the image analysis software)
- Image analysis software for time-lapse movies (we used custom software, PhenoTrack, for automated cell tracking and measurements of protein fluorescent levels in time-lapse movies. The PhenoTrack software was previously described[11,12] and is freely available in our laboratory. Alternatively, other image analysis software that enables cell tracking and protein level quantification can be used.
- PC computer with sufficient serial (RS232), USB2 and FireWire ports to control the various hardware and enable data transfer) ▲ CRITICAL The imaging equipment, software and reagents depicted herein have been used to develop and test the bleach-chase method. However, there are many other comparable equipment types, software and reagents that may be applied to produce similar results. In addition, lower-throughput applications can be performed with less-specialized kits and equipment.

REAGENT SETUP

Cells The cells used to develop and validate this protocol were obtained from the LARC library based on the H1299 non–small cell lung carcinoma cell line[11]. In these cells, proteins were fluorescently tagged at their endogenous chromosomal locations by CD tagging[11–14]. Briefly, a YFP-encoding DNA was designed as an artificial exon and randomly integrated into the genome of target cells by using a retroviral vector. Upon integration within an expressed gene, a fluorescently tagged protein is translated under its native regulation. The tagged proteins were then annotated using 3′ rapid amplification of cloned ends (RACE). Sterility of cell culture work is ensured by spraying 70% (vol/vol) ethanol in the hood, on hands, and on any bottle or tube inserted into the hood.

Cell growth medium For the LARC cells described in the present protocol, use RPMI 1640 medium supplemented with (+) L-glutamine, 10% (vol/vol) fetal calf serum and 0.05% (vol/vol) penicillin-streptomycin, filtered through a 0.45-μM tissue culture filter. This can be filtered and stored under sterile conditions at 4 °C for 2–3 weeks.

Cell freezing medium Cell freezing medium is 7% (vol/vol) DMSO in normal growth medium, filtered through a 0.2-μm filter. It can be stored at 4 °C for 2 weeks.

Fibronectin solution Dilute fibronectin 1:100 in sterile PBS to obtain a 100 μg ml^{-1} solution. Filter the solution with 0.2-μm filters. It can be stored at 4 °C for 4–5 weeks.

EQUIPMENT SETUP

Microscope setup The microscope setup we used is similar to the instructions previously described in Sigal *et al.*[13]. Briefly, install ImagePro and ScopePro on the computer. To connect the microscope components and the software, open ScopePro, configure the software and relate each component to its COMM port. This stage can involve opening ImagePro and then ScopePro. ImagePro will give information about components that are connected inappropriately. The main components to check are the shutters (phase and fluorescence), stage and camera. ▲ CRITICAL Connecting all components correctly is crucial for the system to work properly. The correct connection can be discovered by trial and error. We recommend consulting with a Media Cybernetics expert. After the first successful connection, we highly recommend marking each cable and writing down both the USB port to which it should be connected and the relevant COMM port number. Note that when a prolific USB-to-serial adaptor is used the COMM port it represents will disappear from the device manager if the device attached to it is unplugged. For serial ports from a PCI card, trial and error would be the best way to define the correct port (see TROUBLESHOOTING).

Controlling the camera from the ImagePro software Install the latest version of the camera driver (or an appropriate version for the installed ImagePro), verify that the camera works through its software (not via ImagePro) and then control the camera through ImagePro. It is important to test the camera's compatibility with the ImagePro program before buying the camera.

YuvControl program (available from our laboratory) The YuvControl program contains scripts that enable time-lapse movie acquisition and control via imagePro. First, create a folder named 'current.movie' on the D drive of the computer. In this folder, create the following folders: phase, fluor, fluo2, calibrationStart_fluor and calibrationStart_fluo2. These are the

folders in which the time-lapse images will be saved. Copy the YuvControl file package to your computer. In the ImagePro top ruler, press 'macro', then press 'macro' in the opening list. Change to the folder that contains the YuvControl program package (see TROUBLESHOOTING).

Setup for analysis of the time-lapse movie data Install the image analysis software on your computer. If you choose to work with the PhenoTrack software (custom-written in MATLAB, available from our laboratory), copy it to the MATLAB working path directory.

PROCEDURE

Thawing cells

1| Thaw the vial of frozen cells by adding warm (37 °C) growth medium (up to 1 ml) to the vial.

2| Transfer the thawed cells to a sterile 15-ml or 50-ml tissue culture tube with 10 ml of warm growth medium (repeat Steps 1 and 2 until all cells are thawed).
▲ **CRITICAL STEP** Steps 1 and 2 should be performed relatively quickly.

3| Centrifuge the cells for 2 min at ~300*g* at room temperature (20–25 °C).

4| Discard the supernatant.

5| Resuspend the cell pellet with 10 ml of fresh, warm growth medium.

6| Transfer the resuspended cells to a sterile 10-cm tissue culture dish.

7| Leave in the tissue culture incubator overnight.

8| Repeat Steps 1–7 for all cell clones to be used in the experiment (each clone expresses a different fluorescently tagged protein).

Plating cells for a time-lapse microscopy experiment

9| To plate cells on a 12-well optical plate, first apply 300 µl of fibronectin solution (see REAGENT SETUP) to each well in the plate.

10| Incubate the plate for 45–60 min in the tissue culture incubator.
■ **PAUSE POINT** The plate can be left in the incubator overnight.

11| Wash the plate twice with warm (37 °C) sterile PBS.

12| Trypsinize, with 2 ml of trypsin, the cells from the 10-cm dish (those thawed the day before). Repeat Steps 3–5 and count with a hemocytometer.

13| Plate the cells on the fibronectin-coated 12-well plate (seed about 8×10^6 cells in 2 ml of growth medium per well). Each clone should be seeded in two wells (one well for bleaching and the other for unbleached control).
▲ **CRITICAL STEP** The number of cells that are seeded on the plate should be adjusted according to the experiment length and the rate of cell duplication.

14| Incubate the cells in tissue culture incubator overnight.

Calibrating the fluorescent lamp

15| Open a folder on the computer named 'current.movie' (see EQUIPMENT SETUP).

16| Open the YuvControl program.

17| Place an optical 12-well plate with 2 ml of red RPMI 1640 media on empty wells with no cells. Either change the objective to a ×5 objective or lower the objective apparatus.

18| Insert the plate into the microscope.
▲ **CRITICAL STEP** The plate should be held tight and straight.

19| Switch back to the ×20 objective.

20| Find the bottom focus of the well and elevate the focus plane by 50 µm.

21| Run the calibration macro program.

22| Run calibration on the YFP filter and on the red mCherry filter.

Bleaching
23| Replace the growth medium in each well of the 12-well plate, previously seeded with the fluorescent cells, with 2 ml of complete transparent RPMI 1640 medium (see REAGENTS). Change to a ×5 objective or lower the objective apparatus.

24| Insert the plate into the microscope.
▲ **CRITICAL STEP** The plate should be held tight and straight.

25| Switch back to the ×20 objective.

26| Determine the appropriate bleaching exposure duration required for each protein (see Experimental design). For convenience, this procedure may be performed on the same plate that is used for the time-lapse experiment. However, to prevent unwanted bleaching of the selected FOV, we highly recommend taking the snapshots on a distal area of the plate (at least 700 µm away from the nearest FOV).
▲ **CRITICAL STEP** It is important not to overbleach the cells, as this may cause phototoxicity.
? TROUBLESHOOTING

Acquiring a time-lapse movie
27| Open the YuvControl program: press 'macro' and choose YuvControl. In the opened YuvControl window, you can control your time-lapse movie workflow by choosing the FOVs, the exposure times, the time-lapse interval, the duration of the movie and so on.

28| Open the video microscope camera by pressing 'start preview' on the camera window.

29| Open the transmitted light shutter and focus on the cells.

30| Select FOVs (e.g., choose four FOVs in each well of a 12-well plate) and save the list of FOVs.

31| For each clone, insert the appropriate bleaching exposure time and the nonbleaching exposure time (as determined in previous steps).

32| Insert the appropriate fluorescent-shutter exposure times (for the bleached and unbleached FOVs in the YFP channel, as well as for the mCherry color). Each time you choose an FOV, the exposure times will be added to the information of the FOV. Each FOV is associated with *x*,*y* coordinates, a *z*-plane focus distance and the YFP and cherry exposure times.

33| Insert the exposure time of the phase images and the AF plane images.

34| Insert the desired interval time of the time-lapse movie in the window 'delay(sec)', on the YuvControl window.

35| Determine the number of loops in the 'timeloops' field.

36| Traverse over all FOVs and adjust the focus if necessary.

37| Save the list of points.

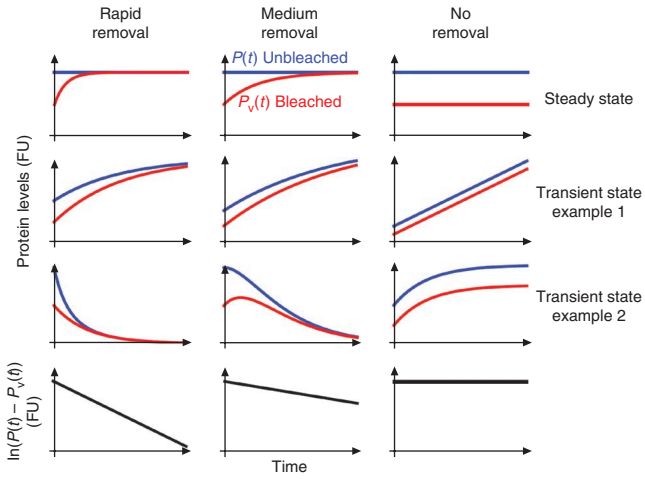


Figure 2 | Schematic results of a bleach-chase experiment. Schematic dynamics for proteins with rapid, medium and no removal. In all the examples the removal rates are constant over time. Red and blue lines correspond to fluorescence levels over time of bleached and unbleached cells, respectively. The top three rows show: bleach-chase dynamics under steady state; transient state 1, in which production increases abruptly at $t = 0$ and then remains constant; and transient state 2, in which production decreases exponentially with time. See ref. 11. FU, fluorescence units.

38| Press 'Run' to start the movie recording. Run the time-lapse movie for at least one round.

39| Pause the movie.
▲ **CRITICAL STEP** Check the 'pause' box in the YuvControl window so that the bleaching round will occur only once.

40| Adjust the bleaching exposure times for the wells that should be bleached.

41| Run the movie for one round of bleaching. The movie loop with the bleaching exposure times can last for about 2–3 h.
▲ **CRITICAL STEP** Press the 'pause' button after pressing the 'Run' button, so that the movie will run only for one round.
? TROUBLESHOOTING

42| After one round, change exposure times back to normal (nonbleaching) exposure times.

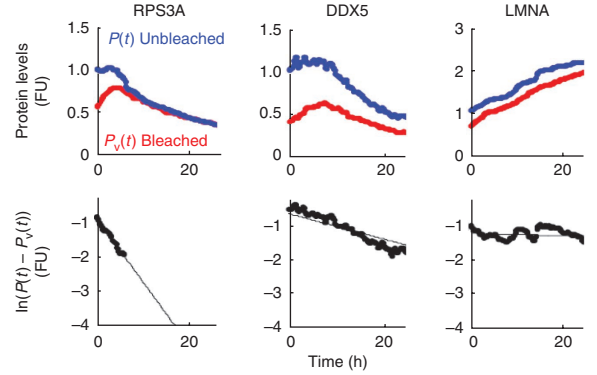
43| Run the movie for the desired amount of time.
■ **PAUSE POINT** Experiments can be analyzed (see the following steps) at your preferred time.

Image analysis and protein half-life measurement

44| Apply image analysis software, such as PhenoTrack, to the time-lapse movies to extract individual cell traces (examples of the expected output are shown in **Figs. 2** and **3**).

45| Compute the protein fluorescence average dynamics over the individual cells in the bleached and unbleached populations.
▲ **CRITICAL STEP** Obtaining robust results requires averaging over a sufficiently large population of cells (we recommend using at least 200 cells).

46| Compute the protein removal, α, by obtaining the slope of the difference between the bleached and unbleached protein fluorescence on a semilogarithmic plot using a linear regression (see equation 4 in **Box 1**, and ANTICIPATED RESULTS). Regression can be performed using Excel, MATLAB or any other parameter-estimation software. When the removal rate, α, is constant, the protein half-life is $T_{1/2} = \ln(2)/\alpha$.
▲ **CRITICAL STEP** The robustness of your measurement should be evaluated because linear regressions may be sensitive to outliers. First, compute α using a nonlinear fit (equation 4)) and compare it with the linear fit results. In case of a large deviation, consider discarding the measurement. Next, obtain an estimate of the measurement robustness by computing a confidence interval of the maximal and minimal removal rates whose mean square error is 5% larger than the optimal fit.
▲ **CRITICAL STEP** Note that the above computations assume that the removal rate is constant over time. One can relax this assumption (see ANTICIPATED RESULTS).



	RPS3A	DDX5	LMNA
$T_{1/2}$ (h)	3.2 ± 0.6	12.9 ± 3.5	31.0 ± 7.0
α (1/h)	0.21 ± 0.04	0.05 ± 0.02	0.02 ± 0.01

Figure 3 | Examples of bleach-chase results. Bleach-chase dynamics and half-lives of three proteins (RPS3A, DDX5, LMNA) measured over 24 h (20-min resolution) in response to camptothecin-induced stress at $t = 0$. The figure was adopted with minor changes from ref. 11. FU, fluorescence units.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Equipment setup	The computer does not communicate with the COMM ports	USB-to-serial adaptor was not installed	Insert and run a USB-to-serial CD (CD is provided with the adaptor)
	The YuvControl software exits with an error message after clicking 'save list' to save the FOVs list	There is no folder named 'current.movie' on the D computer drive	Create a folder named 'current.movie' on the D drive of the computer
26, 41	Fluorescent proteins do not undergo sufficient bleaching	After multiple uses light intensity weakens; the fluorescent light path fiber is damaged	Consider replacing the fluorescent light bulb source or the light path fiber
41	No reduction in fluorescence levels is observed after bleaching when viewing the tiff images	Tiff-viewer software often uses an automated rescaling procedure on the image histogram in order to improve image quality. This may cancel out the bleaching visual effect	Disable the automated rescaling procedure or view the fluorescent images through another software program (e.g., ImagePro, MetaMorph, MATLAB)
	Reduced bleaching effect: a fluorescent protein shows sufficient bleaching in the calibration step, but when running the time-lapse movies the effect disappears or is markedly reduced	The bleaching loop may take up to a few hours. During this time, short-lived proteins, which initially showed significant bleaching undergo rapid degradation. Consequentially, the nonfluorescently tagged proteins are replaced by fluorescent ones. By the time the time-lapse movie begins, the original bleaching signal is substantially reduced	Put the rapidly degrading proteins at the end of the bleaching loop, thus shortening the time between their bleaching and the beginning of the time-lapse acquisition. Alternatively, shorten loop duration by running it on fewer proteins

● TIMING

See **Table 1** for timing information.

ANITICIPATED RESULTS

Cell growth

Under the microscope, cell growth should be comparable to standard tissue culture incubator growth.

Time-lapse movie

Images acquired from the microscope are saved in the relevant folders as tiff images and can be viewed in a variety of programs (e.g., ImagePro, MetaMorph, MATLAB or any tiff-enabling viewer). Note that the images can be viewed individually or sequentially as a movie (see EQUIPMENT SETUP for more details).

Examples of bleach-chase dynamics

Schematic and real data examples of the anticipated bleach-chase results for rapid, medium and slow removal rates are depicted in **Figures 2** and **3**. The graphs produced by bleach-chase can also be used for control purposes. For example, the graphs shown in **Figure 4** indicate a potential artifact, or a removal rate that changes over time.

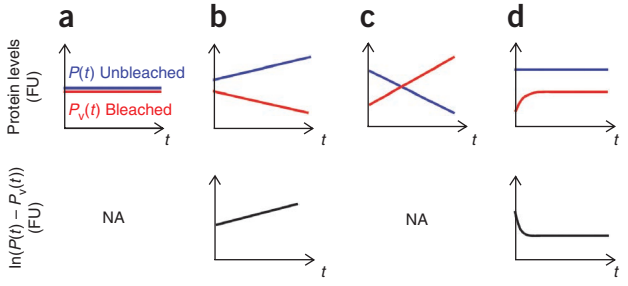


Figure 4 | Examples of dynamics that indicate a potential artifact. (**a**–**d**) The graphs produced by bleach-chase can be used to identify experimental artifacts. (**a**) Bleaching did not reduce fluorescence levels. (**b**,**c**) the bleached and unbleached cells show diverging instead of converging dynamics (**b**) and the fluorescence levels of the bleached and unbleached cells cross each other (**c**). (**d**) Example of bleach-chase dynamics in a case in which the removal rate changes over time (t). This is not an artifact. For details on how to obtain measurements of removal rates that change over time, see ANTICPATED RESULTS. See ref. 11. FU, fluorescence units; NA, not applicable.

Bleach-chase equations summary

The equations underlying the bleach-chase method are summarized in **Box 1**. The *in vivo* protein removal rate, α, is the sum of intracellular degradation, α_{deg}, and the dilution rate, α_{dil} (equation (1)). The removal rate of a protein is inversely related to its half-life (equation (2) holds true if α is constant). Protein levels, $P(t)$, are due to the balance between the protein production rate, β, and the protein removal rate, α (equation (3)). Note that β accounts for both transcriptional and post-transcriptional effects influencing protein production. The nonfluorescent protein fraction can be obtained by subtracting the total fluorescence levels of the bleached cells, $P_v(t)$, from the unbleached cells, $P(t)$ (equation (4)). The slope of decay of the nonfluorescent protein (i.e., $P_v(t) - P(t)$) on a semilogarithmic plot is the removal rate α. Note that this decay depends only on the removal rate and not on the production rate. This equation is derived in the following section.

Bleach-chase mathematical analysis

This section explains how bleach-chase works by deriving equation (4) in **Box 1**, which is used to measure the protein removal rate. First, we assume that the removal rate, α, of a fluorescently tagged protein is constant over time (later we show how to relax this assumption). When the cells are exposed to a brief pulse of light, a fraction of their fluorescent proteins are bleached, effectively turning them into nonfluorescent proteins. Thus, the total protein, P, is a sum of two protein cohorts, one visible by fluorescence microscopy, P_v, and another that is invisible, $\tilde{P}$

$$P(t) = P_v(t) + \tilde{P}(t) \tag{1}$$

The cohort of invisible protein, $\tilde{P}$, is produced only during the bleaching pulse, and it begins to decay immediately afterward, according to the following equation:

$$\frac{d\tilde{P}(t)}{dt} = -\alpha * \tilde{P}(t) \tag{2}$$

Thus, measuring how $\tilde{P}$ changes over time would allow the retrieval of the removal rate, α. However, $\tilde{P}$ is not directly visible via fluorescence microscopy. It can be obtained indirectly by measuring and subtracting P and P_v. Altogether, the difference between the bleached and nonbleached protein fluorescence decays exponentially at a rate that only depends on the protein removal rate (obtained by applying equations (1) and (2)):

$$\frac{d(P(t) - P_v(t))}{dt} = -\alpha * (P(t) - P_v(t)) \tag{3}$$

The solution to this equation is as follows:

$$(P(t) - P_v(t)) = (P(t_0) - P_v(t_0))e^{-\alpha t} \tag{4}$$

Equation (4) in **Box 1** can then be derived:

$$\ln(P(t) - P_v(t)) = \ln(P(t_0) - P_v(t_0)) - \alpha \cdot t \tag{5}$$

One can relax the assumption that the removal rate is constant over time by applying $\alpha(t)$ instead of α in equations (2) and (3) thus replacing $\alpha \times t$ with $\int_0^t \alpha(t')dt'$ in equations (4) and (5).

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1. Ciechanover, A. Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol.* **6**, 79–87 (2005).
2. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–479 (1998).
3. Schwartz, A.L. & Ciechanover, A. The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annu. Rev. Med.* **50**, 57–74 (1999).
4. Nakayama, K.I. & Nakayama, K. Ubiquitin ligases: cell-cycle control and cancer. *Nat. Rev. Cancer* **6**, 369–381 (2006).
5. Pratt, J.M. *et al.* Dynamics of protein turnover, a missing dimension in proteomics. *Mol. Cell. Proteom.* **1**, 579–591 (2002).

6. Alon, U. *An Introduction to Systems Biology* (Chapman & Hall, 2007).
7. Schimke, R.T. & Doyle, D. Control of enzyme levels in animal tissues. *Annu. Rev. Biochem.* **39**, 929–976 (1970).
8. Belle, A., Tanay, A., Bitincka, L., Shamir, R. & O'Shea, E.K. Quantification of protein half-lives in the budding yeast proteome. *Proc. Natl. Acad. Sci. USA* **103**, 13004–13009 (2006).
9. Shwanhausser, B. *et al.* Global quantification of mammalian gene expression control. *Nature* **473**, 337–342 (2011).
10. Yen, H.-C.S., Xu, Q., Chou, D.M., Zhao, Z. & Elledge, S.J. Global protein stability profiling in mammalian cells. *Science* **322**, 918–923 (2008).
11. Eden, E. *et al.* Proteome half-life dynamics in living human cells. *Science* **331**, 764–768 (2011).
12. Cohen, A.A. *et al.* Dynamic proteomics of individual cancer cells in response to a drug. *Science* **322**, 1511–1516 (2008).
13. Sigal, A. *et al.* Generation of a fluorescently labeled endogenous protein library in living human cells. *Nat. Protoc.* **2**, 1515–1527 (2007).
14. Sigal, A. *et al.* Dynamic proteomics in individual human cells uncovers widespread cell-cycle dependence of nuclear proteins. *Nat. Methods* **3**, 525–531 (2006).
15. Hampton, R.Y., Koning, A., Wright, R. & Rine, J. In vivo examination of membrane protein localization and degradation with green fluorescent protein. *Proc. Natl. Acad. Sci. USA* **93**, 828–833 (1996).
16. Li, X. *et al.* Characterization of NFκB activation by detection of green fluorescent protein-tagged IκB degradation in living cells. *J. Biol. Chem.* **274**, 21244–21250 (1999).
17. Zhang, L., Gurskaya, N. & Lukyanov, K. Method for real-time monitoring of protein degradation at the single cell level. *Biotechniques* **42**, 446–450 (2007).
18. Bellanger, S., Demeret, C., Goyat, S. & Thierry, F. Stability of the human papillomavirus type 18 E2 protein is regulated by a proteasome degradation pathway through its amino-terminal transactivation domain. *J. Virol.* **75**, 7244–7251 (2001).
19. Ghaemmaghami, S. *et al.* Global analysis of protein expression in yeast. *Nature* **425**, 737–741 (2003).
20. Jarvik, J.W. *et al.* CD-tagging: a new approach to gene and protein discovery and analysis. *Biotechniques* **20**, 896–904 (1996).
21. Morin, X. *et al.* A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **98**, 15050–15055 (2001).
22. Ciechanover, A. & Schwartz, A.L. How are substrates recognized by the ubiquitin-mediated proteolytic system? *Trends Biochem. Sci.* **14**, 483–488 (1989).
23. Varshavsky, A. The N-end rule pathway of protein degradation. *Genes Cells* **2**, 13–28 (1997).
24. Huh, W.-K. *et al.* Global analysis of protein localization in budding yeast. *Nature* **425**, 686–691 (2003).
25. Liu, H., Krizek, J. & Bretscher, A. Construction of a GAL1-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. *Genetics* **132**, 665–673 (1992).
26. Buszczak, M. *et al.* The carnegie protein trap library: a versatile tool for *Drosophila* developmental studies. *Genetics* **175**, 1505–1531 (2007).
27. Nagai, T. *et al.* A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* **20**, 87–90 (2002).
28. Tsien, R.Y. The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544 (1998).
29. Megerle, J.A., Fritz, G., Gerland, U., Jung, K. & Rädler, J.O. Timing and dynamics of single cell gene expression in the arabinose utilization system. *Biophys. J.* **95**, 2103–2115 (2008).
30. Otsu, N. A threshold selection method from gray-level histograms. *IEEE Trans. Sys. Man Cyber.* **9**, 62–66 (1979).
31. Adams, R. & Bischof, L. Seeded region growing. *IEEE Trans. Pattern Anal. Mach. Intell.* **16**, 641–647 (1994).
32. Eden, E. *et al.* An automated method for analysis of flow characteristics of circulating particles from in vivo video microscopy. *IEEE Trans. Med. Imaging* **24**, 1011–1024 (2005).