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Measuring protein stability in living zebrafish embryos using Fluorescence Decay After Photoconversion (FDAP)

--Manuscript Draft--

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Abstract:	Protein stability influences many aspects of biology, and measuring the clearance kinetics of proteins can provide important insights into biological systems. In FDAP experiments, the clearance of proteins within living organisms can be measured. A protein of interest is tagged with a photoconvertible fluorescent protein, expressed in vivo and photoconverted, and the decrease in the photoconverted signal over time is monitored. The data is then fitted with an appropriate clearance model to determine the protein half-life. Importantly, the clearance kinetics of protein populations in different compartments of the organism can be examined separately by applying compartmental masks. This approach has been used to determine the intra- and extracellular half-lives of secreted signaling proteins during zebrafish development. Here, we describe a protocol for FDAP experiments in zebrafish embryos. It should be possible to use FDAP to determine the clearance kinetics of any taggable protein in any optically accessible organism.
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FDAP invited manuscript

June 24, 2014

Dear Dr. Nguyen,

Thank you for your editorial comments on our manuscript “Measuring protein stability in living zebrafish embryos using Fluorescence Decay After Photoconversion (FDAP)”. Below we address your comments.

Editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please ensure that **all text** in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.
We have changed several sentences in Section 5 to the imperative mood. We also eliminated “should” and “would” where possible and removed the note at the beginning of Section 5.

For example: Step 5 could be moved to the Representative Results.
Multiple reviewers requested further explanation of control experiments, and reviewer #3 specifically asked for the control experiments to appear in the numbered protocol. We therefore created Section 5 to address these important concerns.

3. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
We have changed several sentences to the imperative mood (see response to point #2 above) and include brief notes to clarify subsequent instructions.

4. The Figure Legends should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
We have shortened the figure legends where possible.

Yours sincerely,

Patrick Müller

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Title:

Measuring protein stability in living zebrafish embryos using Fluorescence Decay After Photoconversion (FDAP)

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Keywords:
Fluorescence Decay After Photoconversion (FDAP), protein stability, clearance rate, confocal microscopy, photoconvertible protein, Dendra2, embryo, zebrafish

Short abstract:
Protein levels in cells and tissues are often tightly regulated by the balance of protein production and clearance. Using Fluorescence Decay After Photoconversion (FDAP), the clearance kinetics of proteins can be experimentally measured *in vivo*.

Long abstract:
Protein stability influences many aspects of biology, and measuring the clearance kinetics of proteins can provide important insights into biological systems. In FDAP experiments, the clearance of proteins within living organisms can be measured. A protein of interest is tagged

with a photoconvertible fluorescent protein, expressed *in vivo* and photoconverted, and the decrease in the photoconverted signal over time is monitored. The data is then fitted with an appropriate clearance model to determine the protein half-life. Importantly, the clearance kinetics of protein populations in different compartments of the organism can be examined separately by applying compartmental masks. This approach has been used to determine the intra- and extracellular half-lives of secreted signaling proteins during zebrafish development. Here, we describe a protocol for FDAP experiments in zebrafish embryos. It should be possible to use FDAP to determine the clearance kinetics of any taggable protein in any optically accessible organism.

Introduction:

The levels of proteins in cells and organisms are determined by their rates of production and clearance. Protein half-lives can range from minutes to days[1-4]. In many biological systems, the stabilization or clearance of key proteins has important effects on cellular activity. Modulation of intracellular protein stability is required for cell cycle progression[5,6], developmental signaling[7-9], apoptosis[10], and normal function and maintenance of neurons[11,12]. Extracellular protein stability affects the distribution and availability of secreted proteins, such as morphogens[13,14], within a tissue.

Over the last few decades, protein stability has mainly been assessed in cell culture using radioactive pulse-labeling or cycloheximide chase experiments[15]. In such pulse-chase experiments, cells are either transiently exposed to a "pulse" of radioactive amino acid precursors that are incorporated into newly synthesized proteins, or they are exposed to cycloheximide, which inhibits protein synthesis. Cultured cells are then collected at different time points, and either immunoprecipitation followed by autoradiography (in radioactive pulse-chase experiments) or western blotting (in cycloheximide experiments) is used to quantify the clearance of protein over time.

Conventional protein stability assays have several shortcomings. First, proteins in these assays are often not expressed in their endogenous environments, but rather in tissue culture and sometimes in cells from different species. For proteins whose stability is context-dependent, this approach is problematic. Second, it is not possible to follow protein clearance in individual cells or organisms over time, and the data from these assays reflects an average of different populations of cells at different time points. Since individual cells may have started with different amounts of protein, may have taken up the radioactive label or cycloheximide at different times, or may have different clearance kinetics, such aggregate data could be misleading. Finally, in the case of cycloheximide chase experiments, addition of the protein synthesis inhibitor may have unintended physiological effects that could artificially alter protein stability[16-18]. These shortcomings can be avoided by using Fluorescence Decay After Photoconversion (FDAP), a technique that utilizes photoconvertible proteins to measure protein clearance dynamically in living organisms[19-25] (see **Discussion** for limitations of the FDAP technique).

Photoconvertible proteins are fluorescent proteins whose excitation and emission properties

change after exposure to specific wavelengths of light[26]. One commonly used variant is Dendra2, a "green-to-red" photoconvertible protein that initially has excitation and emission properties similar to green fluorescent proteins, but after exposure to UV light—"photoconversion"—its excitation/emission properties become similar to those of red fluorescent proteins[23,27]. Importantly, new protein produced after photoconversion will not have the same excitation/emission properties as the photoconverted protein, allowing decoupling of production and clearance upon photoconversion and observation of only a pool of photoconverted protein. Tagging proteins of interest with photoconvertible proteins thus provides a convenient way to pulse-label proteins in intact, optically accessible living organisms.

In FDAP assays (**Figure 1A**), proteins of interest are tagged with a photoconvertible protein and expressed in living organisms (**Figure 1B**). The fusion proteins are photoconverted, and the decrease in photoconverted signal over time is monitored by fluorescence microscopy (**Figure 1C**). The data is then fitted with an appropriate model to determine the half-life of the fusion protein (**Figure 1D**).

The FDAP assay described here was designed to determine the extracellular half-lives of secreted signaling proteins in zebrafish embryos during early embryogenesis[19]. However, this approach can be adapted to any transparent model organism that tolerates live imaging, and could be used to monitor the clearance of any taggable intracellular or extracellular protein. Variations of the technique described here have been performed in cultured cells[20,23] and *Drosophila*[22] and mouse[21] embryos.

Protocol:

1. Generating a photoconvertible fusion construct and injecting dechorionated zebrafish embryos

1.1) Generate a functional construct containing the protein of interest fused to a green-to-red photoconvertible protein (see **Discussion**), then use *in vitro* transcription to generate capped mRNA encoding the fusion protein as in Müller *et al.,* 2012[19].

1.2) Use pronase to remove the chorions from about 30 zebrafish embryos at the one-cell stage. Alternatively, manually dechorionate embryos using forceps[28].

Note: Embryos must be dechorionated for subsequent imaging. If desired, embryos can also be dechorionated just prior to imaging.

1.2.1) Make a 5 mg/ml stock solution of pronase from *Streptomyces griseus* in standard zebrafish embryo medium[19]. Rock the solution gently at room temperature for 10 min to allow the protease to dissolve. Aliquot 2 ml into microcentrifuge tubes and freeze at -20 °C.

1.2.2) Transfer one-cell stage embryos to a 5 cm diameter glass or agarose-coated plastic petri dish containing ~8 ml embryo medium. Add 2 ml of thawed pronase stock solution to the dish and incubate at room temperature for 5 min.

1.2.3) Avoid exposing embryos to air or plastic, as contact with either will cause dechorionated embryos to rupture. Fill a 200 ml glass beaker with embryo medium. Transfer the embryos to the beaker by tilting the petri dish while submerging it in the medium.

1.2.4) After the embryos have settled to the bottom of the beaker, pour out most of the embryo medium, then pour fresh embryo medium into the beaker. The mild swirling of the medium pouring into the beaker causes embryos to lose their weakened chorions.

1.2.5) Repeat step 1.2.4.

1.3) Transfer the dechorionated embryos to an agarose-coated injection dish[29] using a glass Pasteur pipette with a flamed tip. Flaming the pipette tip prevents jagged edges from injuring embryos.

1.4) Co-inject the mRNA and a 3 kDa Alexa488-dextran conjugate[29,30] (**Figure 1B**; see **Discussion** for suggested mRNA and Alexa488-dextran amounts). Inject directly into the center of the cell (not the yolk) to ensure even distribution of mRNA and fluorescent dye once cleavage commences.

Note: The Alexa488 signal will be used during data analysis to generate compartmental masks in order to distinguish between intracellular and extracellular fluorescence.

1.5) Transfer injected embryos to a 1 - 2% agarose-coated well of a six-well plastic dish filled with embryo medium. Incubate in the dark at 28 °C until embryos have reached late sphere stage[31] (approximately five hours post fertilization). Check embryos every one to two hours under a stereomicroscope and remove any debris generated by embryos that have died.

2. Mounting zebrafish embryos for photoconversion and imaging on an inverted confocal microscope

2.1) Use a stereomicroscope to identify one to five healthy embryos, and use a glass Pasteur pipette with a flamed tip to remove them from the dish.

2.2) Gently eject the embryos into a microcentrifuge tube containing ~1 ml of melted 1% low melting point agarose in 1x Danieau's embryo medium (see **Table of Materials/Equipment**) (**Figure 2A**).

Note: Agarose should have a temperature between 40 and 42 °C; higher temperatures could damage the embryos.

2.3) Draw the embryos back into the pipette along with some agarose. Gently eject the agarose and embryos onto the cover glass of a glass-bottom dish (**Figure 2B**). Ensure that the thickness of the cover glass is compatible with the objective on the confocal microscope.

2.3.1) Re-use the glass pipette if desired. To clean the residual agarose out of the pipette and prevent clogging, quickly pipette embryo medium up and down. Place a 15 ml tube filled with ~5 ml of embryo medium next to the stereomicroscope for this purpose.

2.4) Use a metal probe to position the embryos so that the animal pole (blastoderm) faces the cover glass. Work quickly since the agarose will solidify in 20 - 30 s. Use the stereomicroscope to monitor the embryos' positions and readjust as necessary until the agarose hardens.

2.5) Repeat steps 2.1 – 2.4 until the desired number of embryos has been mounted.

Note: In a typical experiment, four agarose drops containing four or five embryos each will fit easily on the cover glass. About 16 embryos can be imaged during a single ideal experiment (**Figure 2C**).

2.6) When the agarose has solidified, fill the glass-bottom dish with 1x Danieau's embryo medium.

3. Photoconverting and measuring the decrease of the photoconverted signal

Note: A 25x or 40x water objective is appropriate for the size and refractive index of zebrafish embryos. It is best to use immersion oil with the same refractive index as water rather than actual water, since water will evaporate during the course of the five-hour experiment. Ensure that the immersion oil is designed to be used with a water (not oil) objective.

3.1) Place a large drop of immersion oil on the objective to ensure that the oil film between the objective and cover glass will not break as the stage moves to different embryo positions during imaging. Securely place the glass-bottom dish onto the stage so that the dish will not shift when the stage moves. If possible, use a heated stage at 28 °C, the optimal temperature for zebrafish development.

3.2) Define each embryo's position in the confocal microscope's software package. Adjust the z-depth for each embryo, and attempt to target roughly the same plane in each embryo.

Note: About 30 µm from the animal pole is a good depth since at this depth the enveloping layer of the embryo can be avoided, imaging area is maximized, and light scattering is minimal. A single optical slice with a thickness of ~3.3 µm provides sufficient data; there is no need to acquire a z-stack (see **Section 5**).

3.3) Collect two signals during the experiment: the "green" signal from the Alexa488-dextran conjugate—which will be used during data analysis to isolate extracellular and intracellular fluorescence—and the "red" signal from the fusion protein after it is photoconverted.

3.3.1) Excite Alexa488 using a 488 nm laser, and collect emitted fluorescence between ~500 and 540 nm.

Note: After photoconversion, many green-to-red photoconvertible proteins (*e.g.*, Dendra2) can be excited with a 543 nm laser and emit fluorescence between ~550 and 650 nm. Adjust as necessary based on the photoconvertible protein used.

3.4) Acquire "pre-photoconversion" images, and configure the confocal microscope's software to image each of the previously defined positions (from step 3.2) with the appropriate imaging conditions every 10 or 20 min for a five-hour time course (see **Section 5** and **Discussion**).

3.5) To photoconvert the fusion protein, switch to a 10x objective and expose groups of embryos to UV light from a mercury arc lamp with a ~300-400 nm excitation filter at 100% output for 2 min. Shift the focus along the z-axis to promote uniform photoconversion (see **Section 5).** Ensure that the immersion oil does not drip onto the 10x objective during photoconversion.

Note: The shifting of focus during photoconversion could be automated in order to avoid variability among experimenters.

3.6) Switch back to the 25x or 40x objective immediately after photoconversion. Ensure that the previously defined positions from step 3.2 are still accurate. If the dish shifted during photoconversion, re-define the positions of the embryos.

3.6.1) Start the program created in step 3.4 and allow imaging to continue for five hours. Note the time elapsed between photoconversion and the start of imaging for each embryo.

3.7) Occasionally check on the experiment. Monitor the level of Danieau's medium and add more if necessary. Restart the software if it has stalled.

3.8) In order to determine the background fluorescence values that will be used during data analysis to estimate the asymptote of an exponentially decreasing model, include some uninjected embryos in the experiment. To determine the instrument noise, which will also be used during subsequent data analysis, acquire an image in the absence of a sample.

4. Analyzing the data using PyFDAP

4.1) Visually inspect the time course data sets from each embryo. Discard data sets from embryos that died during imaging, that shifted significantly, that have very low levels of

photoconverted signal, or that contain regions of cells that look unusual and have stopped moving and dividing (typical of injured or sick embryos).

Note: Occasionally, bubbles in the immersion oil or other artifacts will appear in one or two images in an otherwise usable data set. Note any images that contain artifacts; they will be discarded later, and the remaining time points from such a data set can still be analyzed.

4.2) Use the Python-based software package PyFDAP to analyze the FDAP data. PyFDAP calculates half-lives by determining the average intracellular and extracellular red fluorescence intensity in each image and fitting the data with an exponentially decreasing function (**Figure 3**).

4.2.1) Download PyFDAP (see **Table of Materials/Equipment**).

4.2.2) Use PyFDAP to separate intracellular and extracellular photoconverted signal (**Figure 3A,B**). Use the Alexa488 signal, which is strictly intracellular, to create an intracellular mask. Apply this mask to the corresponding red channel image to prevent intracellular pixels from being considered when calculating average extracellular intensity. To measure average intracellular intensity, invert the mask.

4.2.3) In PyFDAP, display the masked images generated in step 4.2.2. Visually inspect these images and discard data sets in which masks do not accurately distinguish intracellular from extracellular space (this should be rare; note that cell membranes are included in images in which extracellular space has been masked, but they could be removed by altering the thresholding algorithm or by introducing a membrane mask (*e.g.*, using membrane-CFP)). Also discard any single images containing artifacts (*e.g.*, bubbles in the immersion oil) identified in step 4.1.

4.2.4) Use PyFDAP to calculate average extracellular and intracellular fluorescence intensities for each image. PyFDAP calculates these averages by summing the intensities of pixels that fall outside of the mask and dividing by the total number of pixels summed.

4.2.5) Fit the fluorescence data (**Figure 3C**) with the following exponential function:

$$c(t) = c_0 e^{-kt} + y_0$$

where t is time post-photoconversion, $c(t)$ is intensity at a given value of t, c_0 is the intensity at $t = 0$, k is the clearance rate constant, and y_0 is the asymptote that the function approaches as fluorescence decreases (**Figure 1D**). y_0 can be constrained based on the measurements in step 3.8[19].

4.2.6) Use PyFDAP to calculate the extracellular and intracellular protein half-lives (τ) from the clearance rate constants (k) using the following relationship:

$$\text{Half-life } \tau = \frac{\ln(2)}{k}$$

5. Control experiments to assess photobleaching, inadvertent photoconversion, and photoconversion uniformity

5.1) Assessing photobleaching

Note: Photobleaching could cause an artifactual decrease in fluorescence intensity that reflects the bleaching properties of the fluorescent protein in addition to the clearance of the protein of interest.

5.1.1) To assess possible photobleaching, perform one set of FDAP experiments with 10 min intervals between imaging and a second set with 20 min intervals between imaging (**Figure 4**). Analyze the data from both sets of experiments using PyFDAP as described in Section 4.

5.1.2) Compare the half-lives from the 10 and 20 min interval experiments. Longer half-lives from 20 min interval experiments indicate significant photobleaching. If the half-lives from both experiments are identical, photobleaching is not a significant concern.

5.1.3) Alternatively, assess photobleaching by acquiring a series of ~30 images immediately after photoconversion. A significant decrease in fluorescence intensity indicates significant photobleaching.

5.1.4) If photobleaching is detected, use lower laser power, decrease imaging time, or consider using a more photostable photoconvertible protein[32].

5.2) Assessing inadvertent photoconversion

Note: Dendra2 can be photoconverted using 488 nm illumination[27]. When exciting Alexa488 with the 488 nm laser as described in step 3.3.1, inadvertent photoconversion and therefore an artifactual increase in the apparent half-life of the protein of interest is possible. However, we and others[33] have found that 488 nm illumination is an inefficient method of photoconversion in zebrafish embryos.

5.2.1) Use the control experiment described in step 5.1.1 to detect inadvertent photoconversion. Compare the half-lives from the 10 and 20 min interval experiments. Shorter half-lives from 20 min interval experiments indicate significant inadvertent photoconversion. If the half-lives from both experiments are identical, inadvertent photoconversion is not a significant concern.

5.2.2) If inadvertent photoconversion is detected, use a lower 488 nm laser power and shorter imaging times to avoid inadvertently photoconverting Dendra2.

5.3) Assessing photoconversion uniformity

Note: If photoconversion is biased toward the animal pole of the embryo, the decrease in fluorescence will be influenced by protein diffusion or cell movement into deeper planes (**Figure 5A**).

5.3.1) To determine whether photoconversion is uniform, express a secreted photoconvertible protein (for experiments with extracellular fusion proteins) or a cytoplasmic photoconvertible protein (for experiments with intracellular fusion proteins). Photoconvert as usual, then acquire a z-stack encompassing most of the blastoderm every 20 min for 80 min.

5.3.2) If photoconversion is biased toward the animal pole, the fluorescence intensity in deeper planes will increase over time due to diffusion or cell movement (**Figure 5B**). If non-uniform photoconversion is detected, focus deeper into the embryos during photoconversion.

Representative Results:

FDAP has been used to determine the half-lives of extracellular signaling proteins in zebrafish embryos[19]. One of these proteins, Squint, induces expression of mesendodermal genes during embryogenesis[34]. Squint-Dendra2 activates expression of mesendodermal genes at levels similar to untagged Squint, as demonstrated by qRT-PCR and *in situ* hybridization assays[19]. Embryos were co-injected with Alexa488-dextran and mRNA encoding Squint-Dendra2 and subjected to the FDAP assay. A decrease in the extracellular photoconverted signal intensity over time is evident (**Figure 4A**). Extracellular intensity profiles from 23 embryos were generated using PyFDAP. The resulting data was fitted in PyFDAP with a first-order clearance kinetics model, and an average clearance rate constant k of 1.00×10^{-4}/s, corresponding to an average half-life τ of 116 min, was determined. Similar intensity profiles and clearance rate constants were obtained when the intervals between imaging were 10 or 20 min, suggesting that photobleaching or inadvertent photoconversion did not contribute significantly to intensity changes (**Figure 4B**).

Figure 1: Fluorescence Decay After Photoconversion (FDAP) overview. A) Workflow of an FDAP experiment. B) Injection of mRNA and a fluorescent dye into a zebrafish embryo at the one-cell stage. Protein is produced from the mRNA as the embryo develops over about five hours prior to imaging. The dye labels cells (green circles). C) The fusion protein is photoconverted using a UV pulse, and the decrease in the intensity of the photoconverted signal over time is monitored. D) The data is fitted with an exponentially decreasing function to obtain clearance rate constants (k) and half-lives (τ).

Figure 2: Mounting zebrafish embryos for FDAP experiments. A) Zebrafish embryos (blastoderm = white, yolk = black) are transferred from embryo medium (blue) into melted agarose (yellow). B) Embryos and agarose are placed onto the cover glass of a glass-bottom dish. Embryos are then manually positioned so that the animal pole faces the cover glass. A cross-section of a glass-bottom dish is shown. C) Schematic overview of a glass-bottom dish with several agarose drops containing four embryos each (view looking down into the dish).

Figure 3: Data analysis using PyFDAP. A) PyFDAP uses the Otsu thresholding algorithm[35] to generate intra- and extracellular masks from the intracellular Alexa488 signal (green). B) Photoconverted signal (red) from an embryo expressing a secreted Dendra2 fusion protein (Squint-Dendra2[19]). Average extra- and intracellular fluorescence intensities were calculated using the masks shown in (A). The space outside of the embryo was excluded from these calculations by discarding pixels outside of the yellow circle. C) PyFDAP screenshot displaying extracellular intensity data from an FDAP experiment (black circles) fitted with an exponentially decreasing function (red dashed line). The extracellular half-life is indicated by the red arrow.

Figure 4: Representative FDAP results. A) A representative embryo expressing secreted Squint-Dendra2 just prior to photoconversion (far left) and 27, 87, and 287 min post-photoconversion. B) To control for photobleaching and inadvertent photoconversion (see **Section 5**), experiments with 10 or 20 min intervals were performed (data from Müller *et al.,* 2012[19]). In PyFDAP, extracellular intensity profiles were generated, fitted with exponentially decreasing functions, and normalized by subtracting the fitted y_0 value from each data point and dividing by the fitted c_0 value. Data from the 10 min (black, n = 11) and 20 min (blue, n = 12) interval experiments were then averaged, respectively. Error bars indicate standard deviation.

Figure 5: Assessing photoconversion uniformity. A) Non-uniform photoconversion of an extracellular protein can lead to an erroneously short apparent half-life if photoconverted protein diffuses into deeper planes over time. B) To determine whether photoconversion is uniform, a z-stack covering most of the blastoderm is acquired at several time points post-photoconversion. Fluorescence intensity will increase in deeper planes over time if photoconversion was non-uniform (note that light scattering causes deeper planes to appear dimmer than higher planes).

Discussion:

The success of an FDAP experiment relies on the generation of a functional photoconvertible fusion protein. Tagging a protein can affect its biological activity and/or biophysical properties, including its localization, solubility, and stability[36-41]. Be prepared to test the activity of several different fusion constructs in order to find one that is active. We have found that changing the position of the photoconvertible protein relative to the protein of interest or using longer linkers (*e.g.*, using the amino acid sequence LGDPPVAT[19]) can enhance the activity of the fusion protein. In the case of signaling proteins, the activity of the fusion protein can be determined by testing its ability to induce expression of target genes[19]. qRT-PCR or *in situ* hybridization provide good readouts of target gene expression[19]. Note that the protocol described here is designed to determine the stability of proteins in the early zebrafish embryo and would require modification to assess protein stability in other contexts.

The green-to-red photoconvertible protein Dendra2[27] has been used successfully in zebrafish FDAP experiments[19] (**Figure 4**), but other options are available[26,42]. To avoid potential artifacts due to aggregation of the fusion protein, choose a monomeric photoconvertible protein. Photoactivatible proteins can also be used in FDAP assays[20-22,26].

Before performing the FDAP experiment, several aspects of the protocol need to be optimized. Inject different amounts of mRNA to determine the lowest amount that provides useable signal after photoconversion; ~50 pg mRNA is a good starting amount. In order to generate meaningful compartmental masks (**Figure 3**), the Alexa488 signal must be bright enough to compete against the signal from the non-photoconverted fusion protein that is constantly produced from the injected mRNA; inject between 0.2 and 4 ng of Alexa488-dextran to find the optimal amount of fluorescent dye. Find the optimal post-conversion imaging conditions and use the same conditions for all experiments with a given construct. Use good quantitative imaging practices[43], and choose an appropriate dynamic range to avoid saturated pixels in the red channel. Determine the appropriate imaging interval for each fusion protein. Proteins with very short half-lives may require more frequent imaging over a shorter total time period. Establish the optimal photoconversion technique based on the organism and photoconvertible protein used. We describe one robust photoconversion method using a mercury arc lamp in step 3.5, but Dendra2 can also be photoconverted with a 405[33] or 488 nm laser[27].

One limitation of this FDAP protocol is that overexpression of the protein of interest is required. Overexpression could affect protein stability, for instance, through abnormal expression of other genes that modify the protein's clearance kinetics[14,44]. If the protein of interest is a signaling molecule, consider performing experiments in the presence of a signaling inhibitor to determine whether blocking expression of target genes affects protein stability. In the future, it may be possible to generate transgenic embryos expressing photoconvertible fusions under the control of endogenous expression elements[45-50]. If transgenic embryos produce sufficient signal, FDAP experiments in a non-overexpression context are conceivable, perhaps using light-sheet microscopy to observe fluorescence decrease in the entire embryo[51].

Possible further applications of FDAP include investigating the mechanisms that regulate protein stability by examining the effects of different perturbations (*e.g.*, expression of putative proteases) or protein modifications (*e.g.*, phosphorylation) on stability. For example, the factors controlling the extracellular stability of Squint are currently unknown. Many secreted developmental signals are internalized by cells[52-55], which could contribute to the clearance of Squint and other ligands from the extracellular space. FDAP experiments in which internalization is blocked might provide information about mechanisms controlling extracellular protein clearance.

In contrast to conventional assays for measuring protein stability[15], FDAP offers a microscopy-based alternative in which the clearance of proteins can be monitored over time within living organisms. Similar methods have been used to monitor protein clearance in model systems other than zebrafish embryos[20-23]. This FDAP protocol has the potential to be adapted to determine the half-life of any taggable protein in biological systems where live imaging is feasible.

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The authors have no conflicts to disclose.

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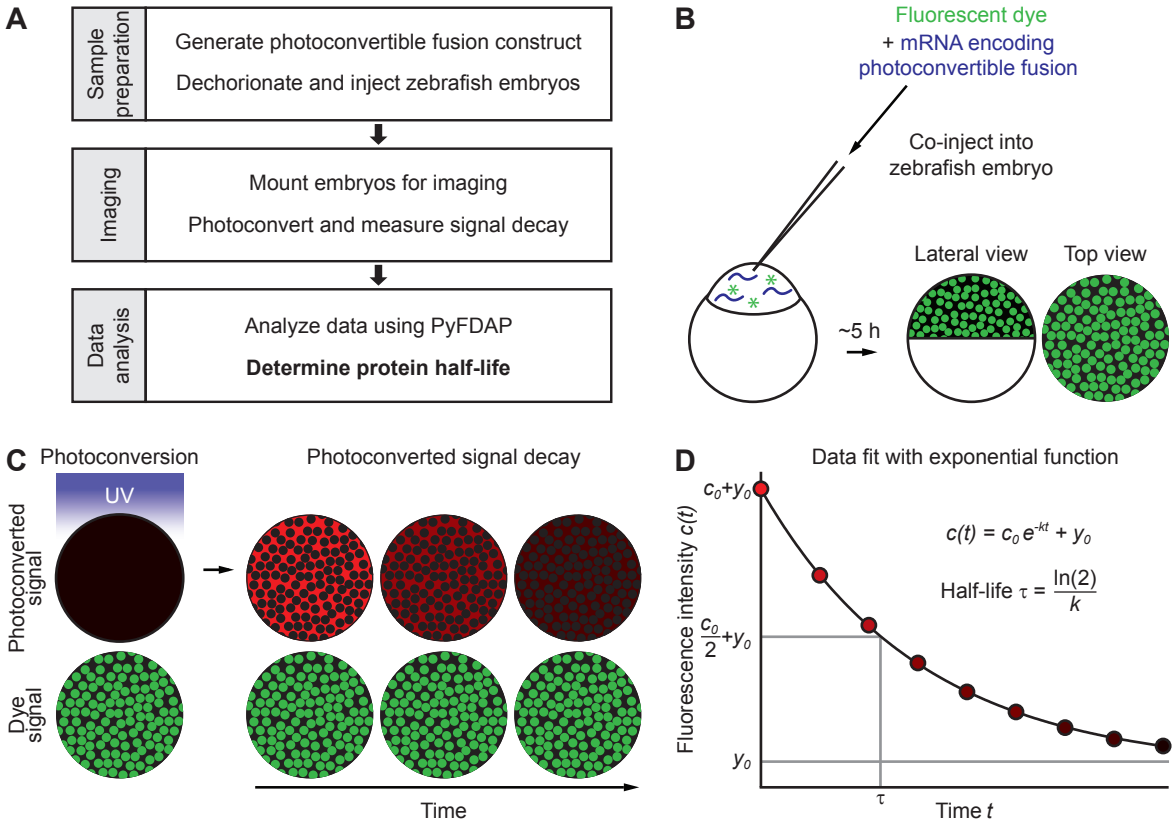

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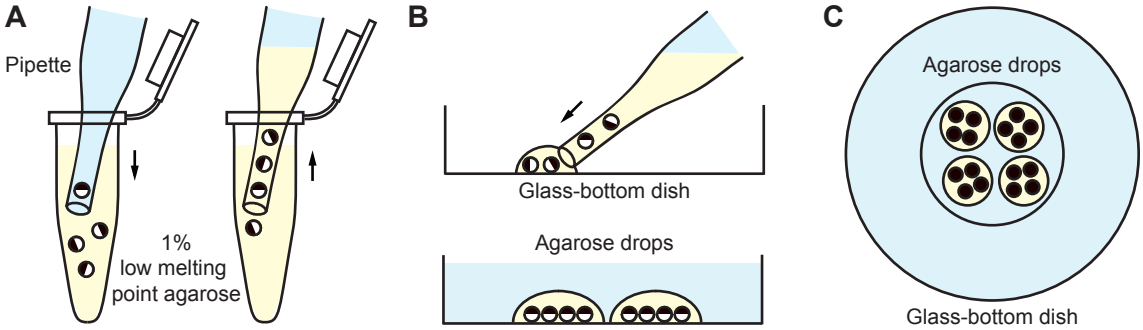

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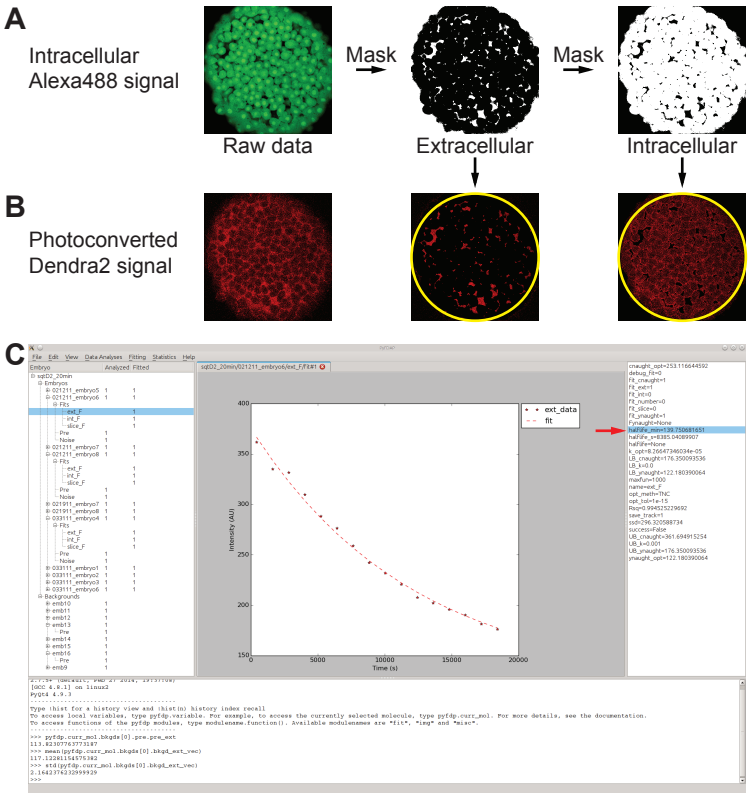

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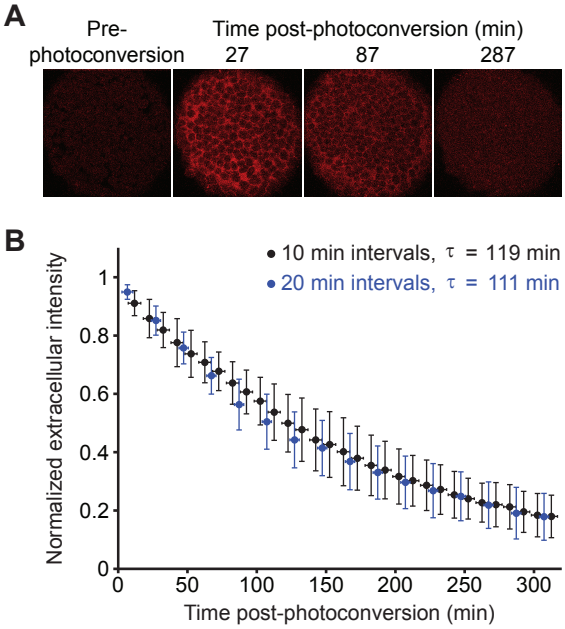

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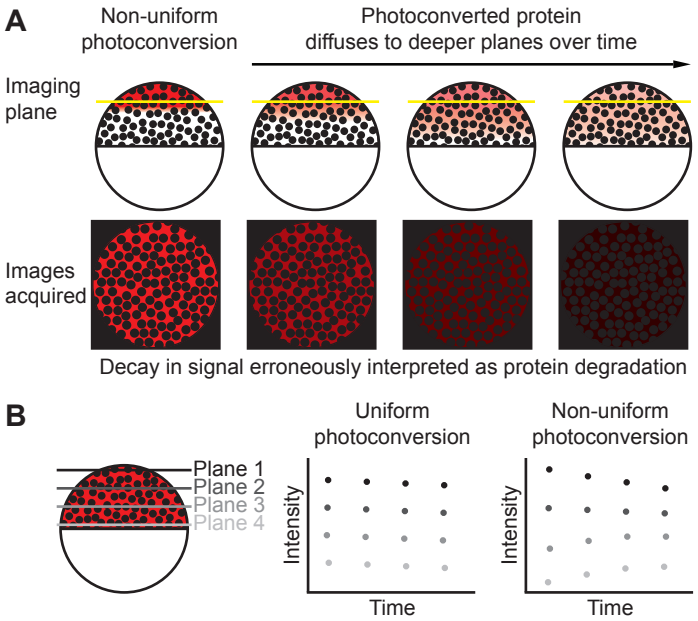

Revised Excel Spreadsheet- Table of Materials/Equipment
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Name of Material / Equipment

PyFDAP (download from the following website: http://people.tuebingen.mpg.de/mueller-lab)
mMessage mMachine Sp6 Transcription Kit (Life Technologies, AM1340)
Alexa488-dextran conjugate, 3 kDa (Life Technologies, D34682)
6-well plastic dish (BD Falcon)
Embryo medium
Protease from *Streptomyces griseus* (Sigma, P5147)
5 cm diameter glass petri dish
200 ml glass beaker
Microinjection apparatus
Stereomicroscope
1x Danieau's medium
UltraPure low melting point agarose (Invitrogen, 16520-100)
Glass Pasteur pipette (Kimble Chase (via Fisher), 63A53WT)
Metal probe
Glass bottom dishes (MatTek, P35G-1.5-14-C)
15 ml tube (BD Falcon) filled with ~5 ml embryo medium
Inverted laser scanning confocal microscope
Heated stage
Confocal software capable of time-lapse imaging
25x or 40x water objective
10x air objective
Immersion oil

Comments / Description

Install and operate using the instructions provided on the PyFDAP website; PyFDAP is compatible with Linux, Mac, and Windows operating
To generate capped mRNA for injection into embryos
Co-inject with mRNA to create intracellular and extracellular masks
Incubate embryos in agarose-coated wells until ready for mounting
250 mg/l Instant Ocean salt, 1 mg/l methylene blue in reverse osmosis water adjusted to pH 7 with $NaHCO_3$
Make a 5 mg/ml stock and use at 1 mg/ml to dechorionate embryos at the one-cell stage
For embryo dechorionation
For embryo dechorionation
For injection of mRNA and dye into embryos at the one-cell stage
For injecting and mounting embryos
Dilute low melting point agarose and perform imaging in this medium; recipe: 0.2 mm filtered solution of 58 mM NaCl, 0.7 mM KCl, 0.4 mM M
For mounting embryos; use at a concentration of 1% in Danieau's medium: add 200 mg to 20 ml Danieau's medium, microwave until dissolve
For mounting embryos; flame the tip to prevent jagged edges from injuring embryos
For positioning embryos during mounting
Use the appropriate cover glass thickness for your objective; part number listed here is for cover glass No. 1.5
For rinsing residual agarose from the Pasteur pipette
A mercury arc lamp, 488 nm laser, 543 nm laser, and the appropriate filter sets are required
To maintain embryos at the optimal temperature of 28 °C during the experiment
Must be able to define multiple positions and automatically image them at defined intervals
Objective for imaging
Objective for photoconversion
Immersion oil with the same refractive index as water

$MgSO_4$, 0.3 mM $CaCl_2$, 5 mM HEPES pH 7.2

d, then aliquot 1 ml into microcentrifuge tubes; aliquots can be stored at 4 °C, re-melted at 70 °C, and cooled to 40 - 42 °C when ready

/ to use

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Article Title: Measuring protein stability in living zebrafish embryos using Fluorescence Decrease After Photoconversion (FDAP)

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FDAP invited manuscript

June 24, 2014

Dear Dr. Nguyen,

Thank you for your editorial comments on our manuscript “Measuring protein stability in living zebrafish embryos using Fluorescence Decay After Photoconversion (FDAP)”. Below we address your comments.

Editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please ensure that **all text** in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.
We have changed several sentences in Section 5 to the imperative mood. We also eliminated “should” and “would” where possible and removed the note at the beginning of Section 5.

For example: Step 5 could be moved to the Representative Results.
Multiple reviewers requested further explanation of control experiments, and reviewer #3 specifically asked for the control experiments to appear in the numbered protocol. We therefore created Section 5 to address these important concerns.

3. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
We have changed several sentences to the imperative mood (see response to point #2 above) and include brief notes to clarify subsequent instructions.

4. The Figure Legends should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
We have shortened the figure legends where possible.

Yours sincerely,

Patrick Müller