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Application of photobleaching for measuring diffusion of prion proteins in cytosol of yeast cells

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Abstract

Measurement of fluorescence recovery after photobleaching (FRAP) is a non-invasive technique for studying protein dynamics in real time in living cells. FRAP studies are carried out on proteins tagged with green fluorescent protein (GFP) or one of its spectral variants. Illumination with high intensity laser light irreversibly bleaches the GFP fluorescence but has no effect on protein function. By photobleaching a limited region of the cytoplasm, the rate of fluorescence recovery provides a measure of the rate of protein diffusion. A detailed description of the FRAP technique is given, including its application to measuring the mobility of GFP-tagged Sup35p in [*psi*$^-$] and [*PSI*$^+$] cells.
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1. Introduction

The last decade has seen a revolution in the use of fluorescent microscopy in living cells brought about by the ability to express proteins with fluorescent tags. The most commonly used fluorescent tag is the green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria* [1]. Development of variants of this protein with different spectral properties has enabled multiple proteins tagged with bright fluorescent signals to be imaged simultaneously [2]. There have been numerous applications of this technology for studying such diverse phenomena as protein diffusion, protein–protein interactions, and protein dynamics in both prokaryotic and eukaryotic cells, as well as whole organisms including *Caenorhabditis elegans*, *Drosophila*, and mice. To achieve optimal imaging for a given sample a variety of different microscopes have been used. The total internal reflectance microscope images the basolateral surface with depth of field of about 100 nm, the confocal microscope images a section from one to several microns, and the wide-field microscope images the whole visual field.

One specific use of GFP fusion proteins is to study protein dynamics by determining the rate and magnitude of GFP fluorescence using the method of fluorescence recovery after photobleaching (FRAP) [3–5]. GFP produces bright, stable fluorescence that does not fade in low intensity light but is irreversibly bleached by high intensity light under conditions in which there is no significant damage of the protein fused to GFP. Thus the movement of non-bleached molecules into a bleached area can be monitored over time. If a GFP-tagged protein is reversibly bound to an immobilized structure, the rate of fluorescence recovery is typically determined by the dissociation rate of the protein rather than its rate of rebinding because the latter rate is usually only diffusion limited and is therefore much faster than the dissociation rate. On the other hand, if the GFP-tagged protein is diffusing freely in the cytosol or in a membrane, the time course and extent of fluorescence recovery provides information about the fraction of freely

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mobile protein and its rate of diffusion. The rate of fluorescence recovery of cytosolic proteins and membrane-bound proteins is governed by three-dimensional diffusion and lateral diffusion, respectively.

The confocal laser scanning microscope is suited for FRAP studies because it provides control over the region of illumination. This is achieved by use of the confocal pinhole which limits the amount of photobleaching in the lateral plane along the optical axis by removing out-of-focus fluorescence. Even with the pinhole, the conventional one-photon confocal fluorescence microscope accurately bleaches a defined area only in two-dimensional space [6,7]. Since the three-dimensional volume of the photobleached area is not well defined, the usefulness of this microscope in determining the true diffusion coefficient of a freely diffusible molecule is limited. Photobleaching in three-dimensional space can be achieved with much greater accuracy by using a two-photon or multi-photon fluorescence microscope because fluorophore excitation occurs only at the focal point of the microscope. This provides a well-defined three-dimensional photobleach volume and, provided that bleaching is done on a very-fast time scale, allows a true diffusion constant of the fluorophore to be measured in solution [7]. This methodology has also been applied to measuring the diffusion constants of GFP-tagged proteins in the cell, but in this case it is necessary to correct for anomalous diffusion due to transient binding to the immobile cytoplasmic matrix or to less mobile cytoplasmic proteins. Another technique for measuring diffusion coefficients is fluorescence correlation spectroscopy. This method, which measures fluctuations in fluorescence caused by diffusion of excited fluorophores in and out of a defined volume, is especially well-suited for rapidly diffusing molecules.

Even though a single-photon confocal microscope is limited in providing true diffusion coefficients, it can be used to measure relative diffusion coefficients of cytosolic proteins provided that identical photobleach settings are used when comparing either different GFP-fusion proteins or the same protein under different conditions. Therefore, even though the time course of the fluorescence recovery is not a single exponential, the half-life of the fluorescence recovery can be calculated to give a relative measure of the diffusion constant. Such an analysis has recently been applied to freely diffusing cytosolic proteins in both yeast and mammalian tissue culture cells [8–11].

FRAP has recently been used to study the mobility of the yeast cytosolic prion protein Sup35p by expressing a full-length construct of this protein fused to GFP. The relative diffusion of this construct in the non-prion and prion forms was measured in [*psi*$^-$] and [*PSI*$^+$] cells, respectively [8]. This article describes in detail the methodology for performing these experiments, which complement biochemical methods such as sedimentation and size chromatography to distinguish between soluble and aggregated material.

2. Experimental method

2.1. GFP-constructs of prion protein

To use FRAP to measure diffusion of prion proteins, constructs of prion proteins have to be tagged with GFP. It is necessary to insure that the GFP-tagging of the protein does not alter its functional properties. In the case of prion proteins, this means that in addition to the native conformation having normal physiological activity, it must also undergo conversion to the prion form of the protein that is able to self-propagate. Since the level of expression is important in prion biogenesis, it is best to use the endogenous promoter to express the protein. Such functional constructs of GFP-tagged prion proteins have been made for Ure2p and Sup35p. With Sup35p, the functional GFP protein (referred to as NGMC) was made by introducing the GFP between the N-terminal and middle domains of Sup35p [8]. Note that the fluorescence signal of the GFP-tagged proteins must be sufficiently bright to allow photobleaching.

2.2. Sample preparation

Several small colonies of yeast cells expressing the GFP-tagged prion protein, which were freshly plated on 1/2YPD medium (0.5% yeast extract, 2% peptone, and 2% dextrose), are put in 10 ml of synthetic medium (SD, 0.7% yeast nitrogen base, 2% glucose) with CSM (complete supplement mixture, Q-BIOgene). The cells are grown overnight at 30 °C with 200 rpm shaking. The absorbance of the cells is measured at 600 nm the following morning, followed by dilution of the cells in 5 ml of medium to an absorbance of 0.1. The yeast cultures are maintained in log phase (absorbance less than 1 at OD_{600}) by periodic dilution with fresh medium until the imaging experiments are performed. Unlike 1/2YPD medium, SD medium has no auto-fluorescence and is therefore suitable for imaging live cells by fluorescence microscopy.

A chamber slide (e.g. Lab-Tek chamber slide) is prepared by first coating the bottom with a 2 mg/ml solution of concanavalin A (Sigma C-5275) for 10 min. The chamber is dried by airflow in the hood, followed by washing with distilled water, and then redrying. An aliquot of cells is then added to the chamber and after 5 min the unbound cells are removed by gentle washing with medium.

2.3. Description of FRAP

Conditions have to be optimized for the specific sample that is to be photobleached; very different bleach conditions and times of image acquisition are used when measuring a freely mobile protein as opposed to a protein bound to an immobilized structure such as chromatin or Golgi. In experiments designed to examine the diffusion of cytosolic GFP-proteins, images have to be collected on a fast time scale to measure the half-life of fluorescence recovery.