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At the Cutting Edge

# Imaging protein behavior inside the living cell

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**Abstract**

The genetically encoded fluorescent proteins (FPs) have transformed studies in cell biology by allowing the behavior of proteins to be tracked within the natural environment of the living cell. Progressively more complex imaging methods are being used to measure the mobility, co-localization and interactions of proteins labeled with the FPs. This review provides an overview of recent developments in live-cell imaging techniques to analyze the subcellular distribution and interactions of proteins in living cells.


*Keywords:* Bioimaging; Quantitative fluorescence microscopy; Green fluorescent protein; Nuclear structure; FRET; FLIM

**Contents**



## 1. Introduction

Over the past decade, the use of genetically encoded fluorescent proteins (FPs) to track the behavior of proteins within the natural environment of the living cell has transformed studies in cell biology. The FPs have become widely used as non-invasive markers in living cells, and their successful integration into living systems is illustrated by the many examples of healthy transgenic mice that carry these markers (Hadjantonakis and Nagy, 2001; Feng et al., 2000; Walsh and Lichtman, 2003). The modification of existing FPs, coupled with the cloning of new color variants from corals has yielded FPs that emit light from the blue to the red range of the visible spectrum (Patterson et al., 2001; Zhang et al., 2002; Matz et al., 1999, 2002; Karasawa et al., 2004). These different color FPs are being used in combination with a progressively more complex set of imaging methods to address the functional recruitment, co-localization and interactions of specific protein partners within living cells, providing an important complement to the biochemical methods that are traditionally used in this analysis (Lippincott-Schwartz et al., 2001; van Roessel and Brand, 2002; Wouters et al., 2001; Zhang et al., 2002). Accordingly, it has become increasingly important to understand the strengths of each of these different imaging methods and how to best choose a method to address a par-

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ticular biological question. This review aims to provide an overview of recent developments in live-cell imaging techniques to analyze the subcellular distribution and interactions of proteins in living cells.

## 2. Imaging methods to monitor protein localization and mobility in the living cells

Digital fluorescence microscopy is a tool that is now readily available to laboratories interested in examining the location of molecular components in living cells. The instruments that are most often available to laboratories are either laser scanning confocal (LSCM) or wide-field (WFM) microscope systems. The observation of living cells using either type of instrument requires a balance between maximizing the signal while limiting potential photodamage to the sample under observation. In this respect, both LSCM and WFM expose the entire specimen under the objective to the excitation light, so it is important to optimize both these systems to minimize exposure to potentially damaging light. The major difference between the two systems is that the WFM collects all the emitted fluorescence from the sample, including the out-of-focus light from above and below the focal plane, whereas the LSCM puts a pinhole in the light path that prevents most of the out-of-focus signal from reaching the detector. The WFM and LSCM systems each have advantages and disadvantages depending on the biological application (Gerlich and Ellenberg, 2003; Andrews et al., 2002).

The use of WFM is limited by the out-of-focus signal reaching the detector, which becomes substantial with thicker specimens (>30 μm), reducing the contrast in the acquired image (Swedlow and Platani, 2002). However, for thin samples, such as cells growing in monolayer, there are advantages to using WFM. WFM is among the most sensitive methods available, allowing minimal exposure of living cells to the excitation light (Gerlich and Ellenberg, 2003; Andrews et al., 2002). Other advantages of WFM include its uniform illumination, unlimited choice of excitation wavelengths, and simplicity. In addition, the out-of-focus signal in WFM images can be either removed or reassigned to its point of origin by using image deconvolution computer algorithms (reviewed by Wallace et al., 2001; Swedlow, 2003). WFM is therefore well suited for monitoring the temporal and spatial dynamics of proteins in living cells using multi-color fluorescence imaging (Fig. 1A). An example of this application comes from studies of Janicki et al. (2004), who developed a system to visualize gene expression in real-time in a living cell model. They used a stable cell line that expresses a multicopy transgene containing an array of lactose operator (LacO) repeats. The binding of cyan FP (CFP)-labeled Lac repressor proteins to the array was used to mark its position in the living cell nucleus. Then, transcription from the array was detected using the viral coat protein MS2 labeled with yellow FP (YFP), which bound specifically to viral translational operators in the nascent transcripts. This system allowed both the morphology of the array and the kinetics of RNA synthesis from the array to be visualized in real-time. Further, by using the expression of other FP-fusion proteins, the association of different histones with the array could also be monitored (Janicki et al., 2004).

For samples thicker or more complex than cell monolayers, the LSCM produces superior images compared to the unprocessed images obtained by WFM (Swedlow and Platani, 2002). However, there are limitations in the use of LSCM in quantitative multi-color imaging. First, there are pixel-to-pixel fluctuations in illumination power found with laser scanning, which can make quantifying signals difficult (Swedlow et al., 2002; Andrews et al., 2002). In addition, care must be taken in acquiring LSCM images since the spectral response and gain can be tuned independently for each of the two or more detectors, which will change their relative sensitivities. Finally, LSCM has a limited number of available excitation laser lines. For example, the standard 458 nm argon ion laser line is commonly used to excite cyan fluorescent protein (CFP, peak excitation of 434 nm), but it is not optimal for this task. The optimal excitation of CFP can be achieved using a frequency-doubled diode laser, which often must be added to existing LSCM systems (van Rheenen et al., 2004).

The major strength of LSCM comes from the ability to rapidly photobleach targeted areas of a cell by repeated scans of user-defined regions of interest (ROI). This capability is exploited in the technique of fluorescence recovery after photobleaching (FRAP), which uses photobleaching of the labeled proteins in a ROI to measure the kinetics of the redistribution of the population of the labeled proteins over space and time (Fig. 1B). The influx of labeled proteins from outside the bleached area is monitored, and plotting the recovery of fluorescence in the ROI provides an estimate of the mobility of the labeled protein population (Fig. 1B). It is important to realize that proteins interact with varying affinity with many other molecules in the cell, so protein diffusion constants determined from FRAP experiments must be carefully interpreted (Phair and Misteli, 2001).

The FRAP approach has provided important insight into the mobility of proteins inside the cell nucleus, showing that some proteins are highly mobile, whereas other proteins, such as histones and structural proteins are not (Misteli, 2001). For instance, the mobility of the glucocorticoid receptor (GR) in the living cell nucleus has been studied extensively using this technique. Studies by Schaaf and Cidlowski (2003) showed that binding of ligand to FP–GR reduced its mobility in the nucleus by increasing its association with other nuclear structures, similar to earlier results obtained by FRAP analysis of the estrogen receptor (Stenoien et al., 2001). Recent studies have established a link between efficient transcription by the nuclear receptors and their proteasome-mediated degradation (Lonard et al., 2000). The FRAP analysis of GR revealed that inhibition of proteasome activity caused the immobilization of a fraction of the receptor in the nucleus, and this effect was inhibited by ligand binding to the receptor (Schaaf and