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journal homepage: www.elsevier.com/locate/fbr

Technical Focus

Total internal reflection fluorescence microscopy of fungi

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ARTICLE INFO

Article history:
Received 29 July 2010
Received in revised form
22 December 2010
Accepted 29 December 2010

Keywords:
Hyphae
Live-cell imaging
Yeast

ABSTRACT

A large number of critical molecular and cellular events occur at the cell surface and cortex; such as cell adhesion, secretion, cytoskeletal interactions, membrane dynamics and cell wall deposition. Investigating such phenomena using many epifluorescence microscopy methods has been challenging due to less than desirable temporal resolution, signal to noise ratio, and phototoxicity. Total internal reflection fluorescence microscopy overcomes many of these difficulties and represents a viable approach for selective visualization of surface regions of fungal and other eukaryotic cell systems.

1. Introduction

Imaging the cellular and sub-cellular events is an important technique to answer many cell biological questions. There is a variety of fluorescence imaging modalities that are commonly used such as widefield, confocal and deconvolution microscopy, as well as relatively new super-resolution microscopy methods. One of the latter methods is total internal reflection fluorescent (TIRF) microscopy. TIRF microscopy has the ability to excite fluorophores in a single, very thin optical section at the cell's surface (Axelrod, 1989; Kramer, 2004). This ability is accomplished through the generation of an electromagnetic field called the evanescent wave. An evanescent wave can be established at the interface of two media of different refractive indices. As light travels from a medium of high refractive index (e.g., immersion oil/cover glass) into a medium of lower refractive index (e.g., aqueous medium), at an angle of incidence that is at or beyond the critical angle (i.e., the incidence angle at which no light passes), an evanescent wave is generated. This wave of energy has a very low penetration depth, usually less than 200 nm, since it dissipates exponentially with distance. The characteristic distance for decay of the evanescent wave is a function of the incident angle of the light beam, its wavelength, and the difference in the refractive index of media on either side of the interface. Thus, this phenomenon provides an excellent signal to noise ratio and low phototoxic effects when visualizing fluorescently tagged molecules that are located at the

Abbreviations: GFP, green fluorescence protein; TIRF, total internal reflection fluorescence.

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doi:10.1016/j.fbr.2010.12.003

surface of the cell or within a narrow region of cytoplasm beneath the plasma membrane (Steyer and Almers, 2001).

TIRF microcopy has been used to investigate a variety of biological processes since its development in the early 1980s (Axelrod, 1981) and with improvements in image acquisition rates, image analysis programs, and commercially available instruments, its use in cell biological investigations has increased substantially in recent years. Studies of particular notice are those using TIRF microscopy to visualize exocytic (Steyer and Almers, 1999; Ohara-Imaizumi *et al.*, 2004; Letinic *et al.*, 2009) and endocytic (Toomre *et al.*, 2000; Merrifield *et al.*, 2002) events, protein and lipid fluidity in the plasma membrane (Axelrod, 1983), cortical cytoskeletal dynamics (Amann and Pollard, 2001; Chan *et al.*, 2009; Feng *et al.*, 2009), signal-transduction events (Sako *et al.*, 2000) and cellular adhesion (Gingell *et al.*, 1985; Lanni *et al.*, 1985; Reichert and Truskey, 1990). To date, however, the majority of studies have been used in investigations of mammalian systems both for *in vivo* and *in vitro* investigations.

In this technical review, we focus on published applications of TIRF microscope to questions of fungal biology in living cells. Such studies have only appeared in the literature within the past 5 years.

2. Case studies

Live-cell imaging of endocytic events in Saccharomyces cerevisiae

Endocytosis is a complex process that requires a cascade of the molecular events, i.e., cargo sorting, membrane invagination, vesicle scission, and vesicle targeting (Kaksonen *et al.*, 2006). Previously, live-cell imaging of clathrin-mediated endocytosis, including measurements of the timing of endocytic events and the order of machinery and cargo recruitment at endocytic sites was shown in mammalian cells using TIRF microscopy (Merrifield *et al.*, 2002). To study the dynamics of cortical clathrin without fluorescence interference from the underlying cellular structure, TIRF microscopy was the best fit due to its ability to visualize events restricted to the cell surface.

To determine the role of clathrin in yeast endoctytosis, TIRF microscopy was also applied to image fluorescently labeled endocytic/cortical patch proteins in *S. cerevisiae* cells (Kaksonen *et al.*, 2005; Newpher *et al.*, 2005; Toshima *et al.*, 2006). For instance, Kaksonen *et al.* (2005) showed colocalization of Clc1-GFP (GFP-tagged clathrin) and Abp1-RFP at the plasma membrane. These studies revealed that clathrin plays a crucial role in efficient initiation of endocytic-site assembly. TIRF microscopy has therefore provided novel insight into the dynamics of receptor-mediated endocytosis in yeast.

Imaging of the splicing of isolated single pre-mRNA molecules in whole cell extracts of S. cerevisiae

The spliceosome is a highly complex and dynamic ribozyme responsible for the excision of introns from nascent eukaryotic transcripts. Due to the complex nature of its splicing machinery, most studies have been done in bulk biochemical assays (i.e., splicing *in vitro* in either cell extracts or partially purified systems) which limit the availability of detailed kinetic information. Crawford *et al.* (2008) described the methodology to visualize the splicing of fluorescently labeled single pre-mRNA molecules in whole cell extracts of *S. cerevisiae* using TIRF microscopy. Their work demonstrated that this single-molecule approach can be adapted to answering questions about spliceosome assembly, kinetics, and disassembly that are not readily addressable using conventional bulk assays and its ability to directly follow biochemical processes in cell extracts.

Live-cell imaging of microtubule dynamics in Neurospora crassa

Uchida *et al.* (2008) used TIRF microscopy to study cortical microtubule dynamics in leading hyphae of *N. crassa* expressing β-tubulin-GFP. In order to address the roles of microtubule dynamics in polarized hyphal growth, the goal of their study was to obtain high spatial and temporal dynamics of four elements of microtubule instability, i.e., polymerization and depolymerization rates, frequency of catastrophe, and duration of pausing, from individual microtubules (Fig. 1). However, the commonly used types of fluorescence microscopy such as widefield, spinning disk, and confocal microscopy, did not provide adequate resolutions required to monitor individual microtubule dynamics in wild-type hyphae of *N. crassa*. Based on the observations of cortical microtubule dynamics using TIRF microscopy, it was found that microtubule polymerization rates in *N. crassa* hypha were much faster than previously reported in those of any other eukaryotic organism which indicates the possibility of microtubule involvement in the rate of hyphal extension and thus the fast cellular growth rates exhibited by *N. crassa*. Additionally, TIRF imaging provided the means to document long distance anterograde and retrograde movements of microtubule fragments within the cortex. Such behavior has been reported in axons but never in fungal hyphae. Finally these workers were able to describe for the first time in fungi the severing of microtubules.

Visualization of F-actin localization and dynamics with live-cell markers in N. crassa

F-actin and associated proteins are commonly localized in the cortex of cells making TIRF microscopy a logical method for capturing their behavior and organization. In a recent work from the lab of Rosa Mouriño-Pérez (Delgado-Álvarez *et al.*, 2010), a detailed study of F-actin and actin-binding proteins was completed in living hyphae of *N. crassa*. In a similar approach used by Uchida *et al.* (2008), TIRF microscopy proved to be advantageous in capturing the dynamics of microtubules with no evidence of phototoxicity. These investigators showed that actin, fimbrin and ARP-3 colocalized to small patches throughout the cortical cytoplasm but forming a concentrated subapical ring. With TIRF microscopy, the ephemeral nature and motility of these small clusters were accurately documented. In addition, the work provided some direct comparisons between image quality obtained from TIRF and confocal microscopy that demonstrated the strength of TIRF microscopy for imaging cortical structures.