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Review

Biomolecular dynamics and binding studies in the living cell

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Dedicated to the late Robert M. Clegg, a great scientist, teacher and friend

Abstract

Isolation and preparation of proteins of higher organisms often is a tedious task. In the case of success, the properties of these proteins and their interactions with other proteins can be studied *in vitro*. If however, these proteins are modified in the cell in order to gain or change function, this is non-trivial to correctly realise *in vitro*. When, furthermore, the cellular function requires the interplay of more than one or two proteins, *in vitro* experiments for the analysis of this situation soon become complex. Instead, we thus try to obtain information on the molecular properties of proteins in the living cell. Then, the cell takes care of correct protein folding and modification. A series of molecular techniques are, and new ones become, available which allow for measuring molecular protein properties in the living cell, offering information on concentration (FCS), dynamics (FCS, RICS, FRAP), location (PALM, STED), interactions (F3H, FCCS) and protein proximities (FRET, BRET, FLIM, BiFC). Here, these techniques are presented with their advantages and drawbacks, with examples from our current kinetochore research. The review is supposed to give orientation to researchers planning to enter the field, and inform which techniques help us to gain molecular information on a multi-protein complex. We show that the field of cellular imaging is in a phase of transition: in the future, an increasing amount of physico-chemical data can be determined in the living cell.

Keywords: Live cell imaging; Fluorescence microscopy; Dynamics; Proximity; Protein–protein interaction

1. Introduction

The strength of microscopic and spectroscopic techniques is based on the property of (particularly long-wavelength) visible light to non-invasively penetrate biological samples. When using fluorescence, due to the Stokes shift the exciting light can be suppressed nearly completely from emission by optical filters allowing for nearly background-free measurements. Modern live cell imaging and microscopy techniques use these advantages and, step-by-step, allow for measuring an increasing range of molecular properties within living cells, adding important, in some cases complementary data to the *in vitro* results. *In vivo* experiments are of particular help when analysing multi-molecular complexes [37,97,146].

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For many enzymes, the situation is comparably simple: these proteins have a well-defined biochemical function which can be directly verified *in vivo* and *in vitro*. When heterologously expressed, the functionally correct folding of enzymes can be estimated by determining their specific activity. The situation is much more complicated for non-enzymatic proteins which function in the cellular context as members of a protein complex. When these proteins are expressed heterologously as single proteins, it remains unclear if they are folded correctly, since correct folding can only be verified when the functionally relevant context within the cell is included into the analysis. The situation becomes even more complex when the proteins not only have one but more functions, potentially at different locations in the cell and/or at different times during the cell cycle. Additionally, many proteins are chemically modified in order to activate, inhibit or degrade them. In general, this modification is not or not correctly obtained in the heterologous system (for proteins of higher organisms in particular not in *E. coli*). Many biochemical procedures respond to this situation by isolating the native active proteins in their cellular context (or as a member of a protein complex) and analysing these isolated structures. Information on the time dependence of protein function during the cell cycle can be obtained by synchronising the cells and isolating the proteins at particular time points. These experiments with synchronised cells are not always optimal: cells run out of synchronisation and, for example, addition of nocodazol (mitotic arrest) modifies the protein composition of particular protein complexes and the modification of specific proteins. During the cell cycle, fast changes in protein composition of complexes are thus difficult to measure *ex vivo*.

1.1. Immuno-staining

Within the cell, the endogenous proteins can be immune-marked and studied when highly specific antibodies are available. However, without support, antibodies are not able to pass the cell membrane. To get antibodies into the cell, the cell membrane must be treated or the antibodies must be micro-injected, both processes strongly affecting the cell. Thus, in general, when antibody labelling is used inside the cell, the cells are fixed: they have the correct internal structure, however they do not live anymore, the experiment is *in situ*, not *in vivo*. In most experiments, the epitope is recognised by a "primary" antibody which can be labelled by biochemical protocols. Mostly, the Fc domain of this antibody is recognised by a commercially available tagged "secondary" antibody which is responsible for detection (it is labelled by a fluorophore, a gold particle or by other means). When multi-labelling of various proteins by different (differently coloured) secondary antibodies is applied, the different primary antibodies must have different Fc domains, they thus must originate from different animals. Classical antibodies are rather large protein complexes. A much smaller binding element (F_{ab} fragments) can be isolated from the antibodies, labelled and used for the cellular studies. An alternative is to isolate and clone the genes of a specific antibody, and transfect the vector into a cell where it expresses the (hopefully active) specific antibody. For this approach it is desirable to work with small single chain (e.g. cameloid) antibodies [168,169] and to express the antibody gene under inducible external control so that the binding can be controlled since antibody binding might interfere with the cellular function of the recognised protein. The broad application of this technology waits for more cameloid antibody clones becoming available so that a larger number of proteins can be specifically recognised.

1.2. Analysis of fluorescently labelled proteins in vivo

The current improvements in imaging and labelling technologies, in particular fusions to fluorescent proteins, make fluorescence microscopy an important tool for quantitative biology [31]. A broad variety of fluorescence spectroscopy techniques has been applied to monitor biomolecular function in cells via changes in spatial proximity, quenching and brightness, spectral shifts or mobility [97]. Mostly, single photon excitation is used. However, near infrared multi-photon excitation laser scanning microscopy, relying on the simultaneous absorption of two or more photons by a single fluorophore, is an optical technique with high spatial and temporal resolution in living cells and, due to its high penetration depth, in particular in tissue [35,63,111,215].

Some cellular processes can be studied *in vivo* without labelling, e.g. by phase contrast microscopy, auto-fluorescence, Raman spectroscopy or NMR. In general however, currently biomolecules are labelled in order to study their properties and function. Proteins chemically labelled with fluorescent dyes can be placed in the cell e.g. by micro-injection, optical transfection [193] or gene gun procedures [110]. The fluorescent dyes however might modify structure and function of the proteins and the properties of the dyes might be influenced by the cellular context; in general this context is not known in detail. Furthermore, micro-injection might influence cellular processes inducing

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