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Advanced fluorescence microscopy methods illuminate the transfection pathway of nucleic acid nanoparticles

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ABSTRACT

A great deal of attention in biopharmacy and pharmaceutical technology is going to the development of nanoscopic particles to efficiently deliver nucleic acids to target cells. Despite the great potential of nucleic acids for treatment of various diseases, progress in the field is fairly slow. One of the causes is that development of suitable nanoscopic delivery vehicles is hampered by insufficient knowledge of their physicochemical and biophysical properties during the various phases of the transfection process. To address this issue, in the past decade we have developed and applied advanced fluorescence microscopy techniques that can provide a better insight in the transport and stability of nanoparticles in various biological media. This mini-review discusses the basic principles of fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) and single particle tracking (SPT), and gives an overview of studies in which we have employed these techniques to characterize the transport and stability of nucleic acid containing nanoparticles in extracellular media and in living cells.

1. Introduction

In many research areas it is important to obtain quantitative information on dynamic properties of molecules and nanoparticulate matter in biomaterials. In cell biology, for example, it has become clear that the cell organisation is a highly dynamic process and that advanced microscopy techniques can aid in unravelling cellular molecular dynamics [1,2]. Microscopy techniques can also be useful in the drug delivery field, where substantial efforts are being made to develop 'intelligent' nanoscopic particles that are capable of efficiently delivering drugs to target cells [3]. In the field of gene therapy, special interest goes to nanoparticles containing nucleic acids, such as plasmid DNA and small interference RNA. Here, the nucleic acids are combined with a helper material, termed a carrier or vector, which facilitates delivery of the nucleic acids to the target cells. Synthetic vectors are typically based on cationic lipids or polymers which can interact with the negatively charged nucleic acids to form particles ('nanomedicines') in the order of 100 nm diameter. However, obtaining efficient nanomedicines is far from evident as they should be capable of overcoming several extra- and intracellular barriers [4]. First of all, the nanomedicines should not aggregate or release the nucleic acids while being suspended in the blood circulation or when traversing the extracellular space. Secondly they should be able to reach the intended target cells in the body and adhere to their plasma membrane to ensure efficient endocytosis. Once internalized, the nanoparticles should be able to escape or at least release their nucleic acids from the endolysosomal vesicles into the cytoplasm.

While new types of carriers are often produced and tested on a trial-and-error basis, research in our group is aimed at increasing our understanding of nanomedicine transport and stability along the entire delivery route in order to develop new and improved vectors in a rational and efficient manner. Several advanced fluorescence microscopy methods have played an important role in this research during the last decade, such as fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) and single particle tracking (SPT). In this mini-review we discuss the basic principles of each of these techniques and give an overview of studies in which we have employed theses techniques to characterize the transport and stability of nucleic acid containing nanoparticles in extracellular media and in living cells.

2. Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) is a well-known fluorescence microscopy technique that has been around since

Abbreviations: CLSM, confocal laser scanning microscope; DOPE, the neutral fusogenic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, the cationic lipid 1,2-dioleyl-3-trimethylammoniumpropane; FCCS, fluorescence cross-correlation spectroscopy; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; SPT, single particle tracking.

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the 1970s [5,6]. FRAP allows to measure the diffusion of fluorescently labeled molecules or particles on a micrometer scale. A typical FRAP experiment consists of three distinct phases, as is depicted in Fig. 1. First, with a low intensity excitation beam, the fluorescence signal is measured coming from the region of interest in the fluorescently labeled sample. Next, with a high power excitation beam, the fluorescent molecules are quickly photobleached in a particular area, typically in the range of 1 to 100 μm in diameter. The photobleaching step creates a local concentration gradient of fluorescent molecules, which will cause net diffusion of the photobleached molecules out of the photobleached area and of intact fluorescent molecules from the surroundings into the photobleached area. The diffusion process after photobleaching is again monitored with a low intensity light beam. By plotting the fluorescence intensity of the photobleached area as a function of time, where $t = 0$ is the time point immediately after photobleaching, one obtains a typical FRAP curve as is shown in Fig. 1. By fitting of a suitable FRAP model to the recovery curve, it is possible to extract the diffusion coefficient D of the fluorescently labeled molecules in that area. Moreover, if a fraction of the molecules are immobile inside the photobleached area, they cannot be replaced by intact fluorophores and the fluorescence intensity inside this area will not fully recover. Therefore, from the asymptotic fluorescence intensity value, it is possible to calculate the local (im)mobile fraction as well.

Although FRAP experiments were at first limited to a few specialized labs, the FRAP technique became gradually more widespread during the 1990s due to the advent of user friendly confocal laser scanning microscopes (CLSMs) equipped with an acousto optic modulater (AOM) or acousto optic tunable filter (AOTF) [7–9]. An AOM or AOTF can act as a very fast laser shutter, allowing the intensity of the scanning laser beam to be modulated on a pixel by pixel basis, giving the possibility to photobleach any kind of user-defined area in the sample. Despite the simplicity to carry out a FRAP experiment on a CLSM, suitable models for accurate and convenient analysis of the FRAP data were missing at that time. Therefore we saw the need in the early 2000s to develop CLSM based FRAP methods that are accurate but straightforward to use and interpret by the non-specialist. One method made use of a circular bleach area [10], while another one was based on the photobleaching of a line profile [11]. The former is especially useful for diffusion measurements in 3-D extended samples, such as extracellular matrices, while the latter is more appropriate for measurements in smaller objects, such as living cells. Both require the use of a low numerical aperture (NA) objective lens to eliminate diffusion along the optical axis in thick specimen, which is not taken into account by the models because of the mathematical complexity. Therefore, we have more recently developed a multi-photon FRAP method which is compatible with high NA objective lenses and takes diffusion along the optical axis into account [12]. A high NA objective lens is a clear asset in optical microscopy since it provides for a better imaging resolution.

In our research, FRAP has been most useful for studying the mobility of macromolecules or nanoparticles in extracellular matrices, such as mucus from cystic fibrosis patients and the vitreous gel from bovine eyes in relation to gene therapy of the retinal pigment epithelium. For example, by studying the mobility of differently sized macromolecules in bovine vitreous, we have found that the hyaluronic acid network in the interfibrillar spaces of vitreous poses an extra sterical hindrance on the diffusing molecules as a function of their size. This was opposite to what we have found for the diffusion of macromolecules in lung mucus of cystic fibrosis patients [10]. Furthermore, we could show by FRAP that attaching hydrophilic polyethylene glycol (PEG) chains at the surface of polystyrene nanospheres can circumvent the binding to fibrillar structures in the vitreous gel and increases their mobility [13]. In addition, we have successfully applied our FRAP methods in collaboration with others for studying the local diffusion of macromolecules in gel systems for time-controlled drug release [14–18].

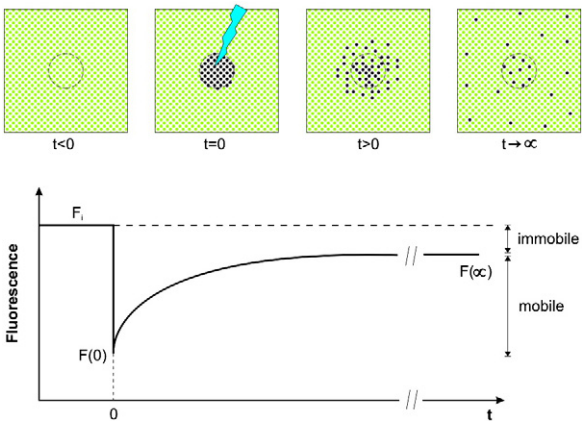

Fig. 1. Schematic representation of a typical FRAP experiment. With an intense laser beam, the fluorescent molecules are quickly photobleached inside a particular area. After photobleaching, diffusional exchange will occur of the bleached and non-bleached molecules, resulting in a gradual recovery of the fluorescence inside the photobleached area. With a suitable mathematical model it is possible to extract the diffusion coefficient and the local (im)mobile fraction of labeled molecules.
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3. Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) is a powerful complementary technique to FRAP that was developed during the same period [19–23]. As illustrated in Fig. 2, an FCS experiment is based on a CLSM type of instrument so that only light from the focal spot can reach the detector. For an FCS measurement the focused laser beam is held stationary at one particular location of interest in the sample. The fluorescence intensity is monitored with very high sensitivity and

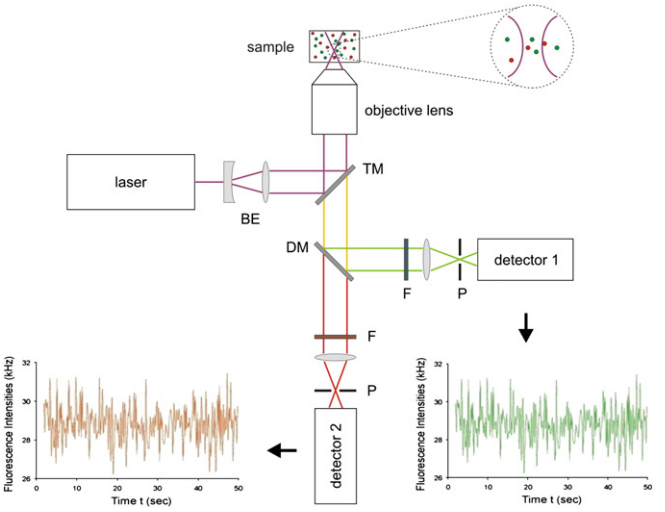

Fig. 2. Schematic representation of an FCS setup. A laser beam passes through a beam expander (BE) and is focused by the objective lens to a diffraction limited volume in the sample after being reflected by the trichroic mirror (TM). The fluorescence light generated by the sample is again collected by the objective lens and is sent to the detectors. A dichroic mirror (DM) in combination with suitable fluorescence emission filters (F) can split up the signal into different spectral channels (e.g. green and red). The confocal pinhole (P) in front of each detector makes sure that only light coming from the focused spot effectively reaches the detectors.
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