New Imaging Tags for Cellular Bioanalysis

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Introduction:

In our desire to understand the many functions that occur within a cell, it is of paramount interest to understand how molecules move and interact within the cellular framework. It is through these comparatively simple processes, that we can ultimately elucidate the complex underlying cellular systems. The easiest way to comprehend these movements and interactions is to visually track molecules in space and time. For this reason there has been much interest during the past three decades to find ways to monitor the activities of cells with simultaneous spatial information. Early cellular studies were conducted using optical microscopy to view the features of a cell, and as studies advanced, stains were used to try to increase the contrast of cellular images. Additionally, 'tags' were developed, which would specifically bind to analytes of interest and act as analyte reporters. A tag can be used to track location, movement, or changes in the target species, and various methods can be utilized to monitor changes in the tags such as EPR, NMR, and fluorescence. Due to this flexibility, the use of 'tags' has acquired great popularity.

While early tags were primarily used to detect stable species such as DNA, proteins and metal complexes, the more recent studies have focused on the detection of unstable/short-lived species such as NO,[1] molecular movements such as that of chromosomes,[2] improvements in sensitivity or efficiency of the reporting-tag.[3] This review will focus primarily on new fluorescence tags and techniques that are being implemented to improve our ability to analyze bio-systems. The two approaches covered in detail will be; (1) to improve detection and localization of the tag and (2) improvements made on tag emission.

Movement Trackers

The use of light microscopy has played a central role in the dynamic analysis of subcellular structures. This has been accomplished by directly tracking the labeled (i.e. "tagged") proteins to investigate their movement within organelles, vesicles, and other subcellular structures. Digital imaging paired with optical and fluorescence microscopy has been used to record protein movements within a cell, and can even be tuned to follow multiple wavelengths simultaneously in three-dimensional space.[2, 4]

In a study conducted by Thomann and co-workers, the aim was to characterize the movement of chromosomes in yeast cells, and model the various chemical forces that drive mitosis.[2] This was successfully accomplished by labeling the chromosome with a green fluorescent protein (GFP) tag, which is used for its specificity and strong binding affinity to the target TET repressor (TetR). Individual chromosomes are labeled with GFP (Figure 1),[5] and their spatial movements can be recorded digitally. The challenge is to track, with high precision, multiple GFP tags exhibiting varying degrees of separation in three-dimensional space.

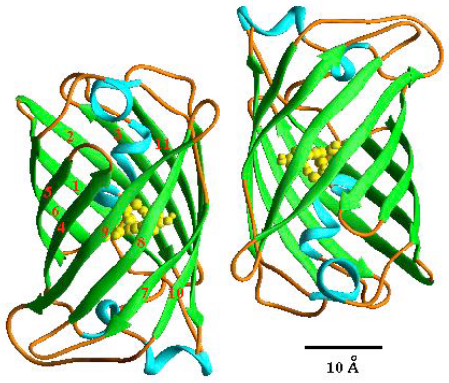

Figure 1. The overall shape of the protein and its association into dimers. Eleven strands of sheet (green) form th walls of a cylinder. Short segments of helices (blue) cap the top and bottom of the b-can' and also provide a scaffold for the fluorophore which is near geometric center of the can.[5]

Thomann et al., as well as other groups, are ultimately trying to understand the movement of chromosomes within budding yeast cells throughout mitosis[6, 7]. The strategy utilized to image the chromosome movements is to track the chromosome and the spindle poles simultaneously. This is essential in determining whether the movements are due to kinetochores (brush-like filaments located in the centromere of the chromosome), or motion resulting from the nucleus under influence of the astral microtubules. The spindle serves as a center reference point to eliminate drift caused by independent kinetochores movement.

The data treatment in the acquisition of high-resolution localized tags is handled through a series of mathematical fits and filtering aimed at improving the resolution between neighboring spots. The algorithm is run independent of the detection and is compiled for each frame of data. The resulting data is then extracted to show the movement of the chromosomes. This study shows that it is possible to pinpoint the location of tags with a 5-10 nm margin of error. When the signal to noise ration is 20 or higher, it is possible to differentiate spots separated by as little half the Rayleigh distance utilizing this algorithm. The Rayleigh distance is defined as the distance beyond which the radiation pattern consists primarily of diffracted energy. However, at this time it is not possible to distinguish between spots that are separated by less than the Rayleigh distance apart (which is approximately 0.23 um) unless the signal to noise ratio is greater than 5.

Molecular Interactions in live cells

Another interest in bioanalysis is to determine and characterize the interactions of molecules within living cells. Kumar and coworkers are probing the interactions (dimerization or oligomerization) between multiple G-protein-coupled receptors (GPCR), and how this affects their encoding from genes in the human genome.[8] Fluorescence resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FCS) are used to detect the fluorescent emission of tagged receptors from live cells, cultured cortical neurons, and brain slices. Insight into the functional diversity within a cell is achieved via protein-protein interactions through oligomerization of GPCR.[9, 10] However, very little is known as to whether this occurs through simple dimer interactions or if higher order oligomers are necessary, and as a consequence, little is known about the stoichiometry of ligand-receptor reactions or the specificity of homo- and hetero-oligomer interactions. To probe this, a two-color FRET probe can be used. Two color

probes have an advantage over antibody probe methods because the localization is very well defined in relation to the binding site.

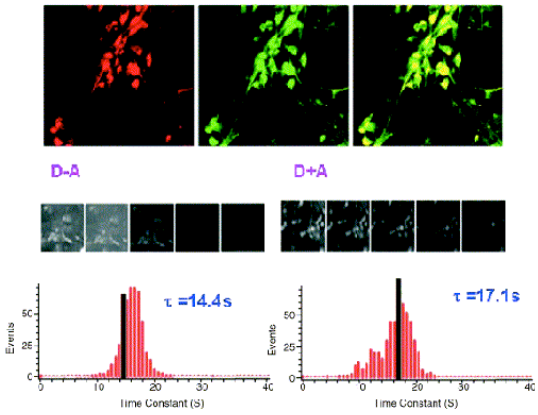

Figure 2. pbFRET showing heterodimerization of endogenous D1R and D5R in primary cultured striatal neurons. The top panel illustrates neuronal localization of D1R (green), D5R (red) and their colocalization (yellow). The middle two panels illustrate the delay in photobleaching of fluorescein-labeled receptors in the presence of Cy3. Pixel analysis of membrane regions plotted as a histogram of photobleaching time (τ) is shown in lower two panels.[8]

Due to the low concentration of fluorescent probes utilized, it becomes important to consider the potential analytical danger of photobleaching. Photobleaching occurs when the overall signal is irreversibly reduced by light induced reactions, which cause the fluorescent species to change conformation or chemical structure in such a way that reduces the photo-stability of the tag. Therefore, to reduce the potential of photobleaching, excitation of the fluorescence is done using a single photon excitation process produced from an argon-ion laser source.

Another strategy to resolve whether GPCR oligomerizes, is to take advantage of the photobleaching process by utilizing photobleaching-FRET (pbFRET), developed by Jovin's group.[11] Hundreds of endogenous receptors can be expressed within a single neuron, so this makes pbFRET very demanding on cultured cortical neurons. Therefore, these experiments are conducted on a fixed specimen, and the experiment that is exemplified utilizes rat-cultured neurons as shown in fig.2.[8] The results illustrate hetero-oligomer formation of the dopamine receptors, and shows promising results for future imaging of this complex system. Further work may lead to a better understanding of receptors, binding effects of antagonists, and the dynamics of ligand-ligand conformational changes.

Ultrabright chromophores

The two approaches described previously deal primarily with improving the localization of a tag as a means to enhance the ability of tracking the changes within the cells. However,

another major challenge to imaging biosystems is the brightness of a tag. The quantum efficiency of tags can be quite low, which makes it very difficult to image the biosystems in vivo. Stellacci and co-workers, have developed a relatively easy method to produce an ultrabright supramolecular tag offering a dramatic increase in quantum efficiency compared to single chromophores, and can be tailored to bind to varying target species in various solvents. [3]

The new tag, chromophore-1-coated nanoparticles (1-Np), (shown in Figure 3) is composed of a metal nanoparticle core with a completely self-assembled layer of chromophores surrounding the particle. This conformation allows for ~2500 chromophores to be packed within a volume less than a 10 nm sphere. The result is an ultrabright nanobeacon that is less susceptible to photobleaching and is ~2500 times more efficient than a single chromophore.

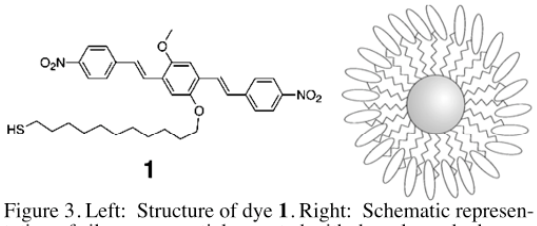

Figure 3. Left: Structure of dye **1**. Right: Schematic representation of silver nanoparticles coated with densely packed chromophores.[3]

1-Np is synthesized by reducing $AgNO_3$ with $NaBH_4$ in the presence of chromophore-functionalized alkylthiol ligands, and is made with a 71% yield from a slightly modified procedure reported by Kim et al.[12] The absorption and fluorescence spectra of the free chromophore and 1-Np in solution were compared, and it was found that they were nearly identical. To use these nanoparticles as tags the synthesis cocktail is tailored to include a second alkylthiol ligand that is specifically functionalized for the target species.

In Stellaci's study, a mixed tag containing the chromophores and 11-mercaptoundecanoic acid was made, which yielded fluorescent particles that are soluble in a mixture of water/ethanol (95:5 vol/vol), and were also tested via absorption and fluorescence to try and determine if their quantum efficiency had changed appreciably from the mono-ligand particle. It was found that there was little difference in the mixed-ligand particle versus the mono-ligand particle. In future

studies these nanoparticles will be implemented as tags, and promise to yield high-resolution images of target species from small concentrations of tags due to the high quantum efficiency.

Summary

Understanding of biological systems requires an in-depth comprehension of the way molecules interact and move within the cells. Several approaches and techniques are available to image and improve understanding of the events occurring within a cell. One way to improve our knowledge of the biosystems is by improving our techniques for localization of the tags with respect to the target of interest, as represented by the work by Thomann et al and Kumar et al.[2, 8] Another approach is to try and improve the tag quantum efficiency in order to minimize the need for high numbers of tags, as illustrated by Perry's group. Due to these and other advances in the field of imaging for bioanalysis, a much clearer picture of three-dimensional movements of molecules within cells is revealed.

References

1. Nagano, T.; Yoshimura, T. *Chem. Rev.* **2002**, *102*, 1235-1269.
2. Thomann, D.; Rines, D. R.; Sorger, P. K.; Danuser, G. *Journal of Microscopy* **2002**, *208*, 49-64.
3. Stellacci, F.; Bauer, C. A.; Meyer-Friedrichsen, T.; Wenseleers, W.; Marder, S. R.; Perry, J. P. *J. Am. Chem. Soc.* **2003**, *125*, 328-329.
4. Bornfleth, H.; Edelmann, P.; Zink, D.; Cremer, T.; Cremer, C. *Biophys. J.* **1999**, *77*, 2871-2886.
5. Yang, F.; Moss, L. G.; Phillips, G. N. *Nat. Biotechnol.* **1996**, *14*, 1246-1251.
6. Thompson, R. E.; Larson, D. R.; Webb, W. W. *Biophys. J.* **2002**, *82*, 2775-2783.
7. Netten, H.; Young, I. T.; van Vlient, L. J.; Tanke, H. J.; Vroljik, H.; Sloos, W. C. R. *Cytometry* **1997**, *28*, 1-10.
8. Kumar, U.; Baragli, A.; Patel, R. C. *Trends in Analytical Chemistry* **2003**, *22*, 537-543.
9. Rocheville, M.; Lange, D. C.; Kumar, U.; Patel, S. C.; Patel, R. C.; Patel, Y. C. *Science* **2000**, *288*, 154-157.
10. Bouvier, M. *Nature Rev. Neurosci.* **2001**, *2*, 274-286.
11. Kubitscheck, U.; Kircheis, M.; Schweitzer-Stenner, R.; Dreybrodt, W.; Jovin, T. M.; Pecht, I. *Biophys. J.* **1991**, *60*, 307-318.
12. Kang, S. Y.; Kim, K. *Langmuir* **1998**, *14*, 226-230.