Long-life fluorescent imaging of cells in real time

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A new family of chemical labels with much longer lifetimes than traditional fluorophores are key to a new biological imaging method: time-resolved emission imaging microscopy.

A fundamental understanding of the mechanisms and processes that control biological systems relies on technological achievements in real-time imaging. A very common imaging modality for studying biological systems is fluorescence microscopy, which benefits from a high spatial and temporal resolution in conjunction with acute sensitivity.^{1,2} A major advantage of fluorescence microscopy is an ability to span magnitudes of length-scale, enabling scientists to visualize whole organisms at one end of the scale right down to single-molecule interactions at the other.

Confocal (and two-photon) microscopy is becoming an increasingly commonplace technique for a multitude of applications, especially for studying cells and tissues in detail. Whatever the application, a fundamental requirement is that of targeting biological entities using stable fluorophores. This is usually achieved by conjugating (joining) the fluorophore directly to an antibody, known as immunofluorescent labeling. Specific biological features can then be identified by the antibody and visualized by exploiting the emissive properties of the fluorophore.³

When imaging complex samples such as tissues, nonlabeled structures, including matrix proteins, frequently create unwanted background interference—known as autofluorescence—that ‘swamps’ the primary signal. This can prevent the acquisition of interpretable images. One way around this would be to make use of differences in the timescale of the emission from the probe and the background. But commercially available fluorophores have inherently very short fluorescence lifetimes (3.6ns, for example, in the case of fluorescein), similar to that of the autofluorescence.

Recently we developed a novel class of luminescent platinum (Pt) (II) labels for biological imaging.⁴ The labels are constructed from a terdentate molecule containing a centrally located

Figure 1. Confocal images of live human fibroblasts, human melanoma cells, and Chinese hamster ovary cells with regions of Pt(II) label accumulation identified. Cells were incubated for 5min, washed with phosphate-buffered saline, and imaged using an excitation wavelength of 488nm. Scale bar = 10μm. (Reprinted with permission.⁴)

platinum ion flanked by two pyridine rings and a phenyl group. Importantly, the luminescent lifetimes of these molecules are measured in microseconds rather than nanoseconds. The structure of the molecule is important for enabling extended lifetimes. In particular, a carbon-platinum bond at the center of the molecule is key. This very strong but short bond directly contributes to the rigidity of the overall structure and in turn the photostability of the label. Thus, one advantage of these molecules over conventional organic labels is a long emission lifetime due to a triplet excited state rather than a singlet state (as the formally spin-forbidden $S_0 \leftarrow T_1$ transition is promoted by the spin-orbit coupling associated with the platinum atom). In addition, these labels have synthetic versatility (they are both easy to make in the lab and easy to alter) as well as chemical and photochemical robustness.

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Figure 2. Time-gated cellular imaging. Live CHO cells were labeled with Pt(II) and imaged in the presence of a solution of fluorescein. Images were taken at 0ns (left) and 10ns (right) after a 355nm laser-excitation pulse (pulse length 0.6ns). (Reprinted with permission.⁴)

Development of Pt(II) complexes has enabled the realization of a fluorescence-based imaging technique called time-resolved emission imaging microscopy (TREM). TREM is similar to fluorescence lifetime imaging microscopy but uses much longer timescales of hundreds of nanoseconds to microseconds. These timescales offer improved discrimination through the much larger changes in lifetime and allow time-gated experiments to distinguish the labels from short-lived autofluorescence.⁵ TREM does not require fast excitation or detection methods. Indeed, it can be performed with nanosecond lasers and slower gated detectors. However, its development has long been hampered by the lack of cell-permeable, nontoxic luminescent probes with lifetimes in excess of 100ns under ambient conditions.

We have therefore investigated the suitability of Pt(II) complexes for live cell imaging.⁴ We demonstrated that direct labeling of human fibroblasts, human melanoma cells, and Chinese hamster ovary (CHO) cells can be achieved by incubating cells with micromolar concentrations of the labels (see Figure 1). Intracellular accumulation was observed within 5min under diffusion control, and the compound was not toxic over a 24h period. One- and two-photon excitation revealed specific labeling of the nuclei of all cells as well as a preferential affinity for DNA and RNA structures. Thus, these remarkably stable complexes satisfy the fundamental requirements for TREM, which include a high emission quantum yield (60–70%), a long emission lifetime, low toxicity, and high cell permeability.

Combining the properties of Pt(II) labels with TREM makes several exciting applications possible. For example, one can readily discriminate between positively labeled biological structures and background autofluorescence by distinguishing between long- and short-lived species. Figure 2 shows a time-gated cell-imaging experiment in which CHO cells were labeled with Pt(II) and imaged in the presence of a solution of fluorescein. Immediately after a laser excitation

pulse, an image was taken at 0ns. Little information can be resolved initially from the micrograph because the fluorescein emission has swamped the image. However, the cells can be observed 10ns after the laser excitation pulse due to the Pt(II) label, which continues to emit light long after the emissive decay of the fluorescein.

In summary, this work shows the power of combining long-lived luminescent probes with TREM for biological imaging and the suitability of this technique for lifetime studies of complex biological structures. We are now generating Pt(II)-conjugated secondary antibodies that will be used in conjunction with monoclonal antibodies for the specific study of biological structures in tissues prone to autofluorescence, in particular skin and nerve.

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References

1. N. Johnsson and K. Johnsson, *Chemical tools for biomolecular imaging*, **ACS Chem. Biol.** **2**, pp. 31–38, 2007.
2. L. Bachmann, D. M. Zezell, A. D. Ribeiro, L. Gomes, and S. A. Ito, *Fluorescence spectroscopy of biological tissues—a review*, **Appl. Spec. Rev.** **41**, pp. 575–590, 2006.
3. K. A. Teare, R. G. Pearson, K. M. Shakesheff, and J. W. Haycock, *Alpha-MSH inhibits inflammatory signalling in Schwann cells*, **Neuroreport** **15**, pp. 493–498, 2004.
4. S. W. Botchway, M. Charnley, J. W. Haycock, A.W. Parker, D. L. Rochester, J. A. Weinstein, and J. A. G. Williams, *Time-resolved and two-photon emission imaging microscopy of live cells with inert platinum complexes*, **Proc. Natl. Acad. Sci. USA** **105**, pp. 16071–16076, 2008.
5. R. R. de Haas, R. P. M. van Gijlswijk, E. B. van der Tol, J. Veuskens, H. E. van Gijssel, R. B. Tjzens, J. Bonnet, N. P. Verwoerd, and H. J. Tanke, *Phosphorescent platinum/palladium coproporphyrins for time-resolved luminescence microscopy*, **J. Histochem. Cytochem.** **47**, pp. 183–196, 1999.