## ***Field:* Chemistry/Biochemistry**

## ***Planning Group Members:***

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## ***Session Topic:***

***Emerging approaches for real-time molecular and cellular imaging***

In the research fields of Biological Chemistry, Chemical Biology, Bioorganic Chemistry, and Bioinorganic Chemistry in the post-genome sequencing project era, not only studies to unravel the role of biomolecules in cells, but also the techniques to utilize their function are needed. To clarify the function of macromolecules such as nucleic acids, proteins and carbohydrates inside the cell, it is necessary to develop new analytical methods. Recently, monitoring the dynamic phenomena of small molecules and macromolecules in living cells was achieved by the improvement of fluorescent probes and fluorescence microscopy. Investigations of the sub-cellar localization and the intermolecular interactions of single molecules are now possible. The technology to specify the function, relations, interactions and structural features of biomolecules has also been promptly improved in these days.

It is expected that the ability to investigate the role of biomolecules inside the living cell will be a starting point for more extended research. One important field could be the development of medicinal chemistry. Identification of certain molecules directly related with a disease or a cell surface receptor will probably allow a molecular-based protection from infection or tumor metastasis. Furthermore, in contrast to conventional passive drug targeting by physicochemical features, new drug delivery systems with active targeting by recognition devices to deliver medicine involving nucleic acids and proteins into target cells will show more and more progress in the future. Especially for the development of gene delivery systems using a non-viral vector, understanding of the intracellular trafficking is very important to enhance the transfection efficiency of plasmid DNA or siRNA. Peptides and oligosaccharides would be useful biomolecules to control sub-cellular localization of nucleic acids.

Understanding and controlling the function of the cell will in the future certainly lead to a much more detailed elucidation of living systems and the human body. Frontier scientists not only in biochemistry and chemistry, but also in all fields of science are now exciting the pioneering studies going on in cell and biomolecule research.

The purpose of this session is to introduce and discuss the currently *emerging approaches for real-time molecular and cellular imaging*, which as an analytical tool play a key role for further progress in chemical biology and *in vivo* chemistry.

*Chemistry/Biochemistry*
*Planning Group Members: Günther Knör and Takehiko Wada*

***Emerging approaches for real-time molecular and cellular imaging***

***Speaker:***
***Shiroh Futaki, Kyoto University***

## 1. Introduction

Biological membranes are hydrophobic barriers to separate the insides and the outsides of the cells. It is therefore difficult to deliver bioactive molecules with a high hydrophilicity, such as proteins and nucleic acids, into cells. On the other hand, the microscopic observation of fluorescently labeled molecules in the cells is one of the most powerful methodologies for the analysis of the intracellular molecular interplays. For the observation of the intracellular proteins, one of the most popularly employed methods is to transfect cells with the plasmids coding the tandem sequences of the proteins of interest and proteins that yield fluorescence. The eventual expression of the fusion proteins enables us to observe the behaviors of the proteins in the cells. However, if exogenous proteins that are chemically modified with fluorescence moieties can directly be brought into cells, the availability of fluorescent labeling with various properties will expand the horizons of the in-cell molecular imaging.

Recently, a novel strategy has been presented to use membrane-permeable peptide vectors to deliver exogenous proteins into cells. Short peptide segments derived from HIV-1 Tat and *Drosophila* Antennapedia homeodomain proteins are among the representatives of these peptides (Table). By conjugation of these peptides either chemically or genetically to cargo proteins, the successful delivery of various proteins into cells has been achieved to control cellular functions. Not only for protein delivery, this strategy has been applied to the delivery of various molecules with wide ranges of molecular sizes and physicochemical properties, such as small molecular weight compounds, oligonucleotides, magnet beads, and even liposomes, to introduce fluorescently labeled molecules in the cells. However, because the majority of these molecules are taken up by the cells using endocytosis and trapped in endosomes, low efficiency of their translocation into cyotsol hampers the observation of the molecules in cytosol. Here we introduce a new approach of the intracellular delivery using a peptide vector in the presence of counteranions, which may overcome this shortage.

***Table***. Examples of membrane-permeable arginine-rich peptides

| | |
|---|---|
| HIV-1 Tat-(48-60) | GRKKRRQRRRPPQ |
| oligoarginine | Rn (n=7-11) |
| Antennapedia-(43-58) (penetratin) | RQIKIWFQNRRMKWKK |

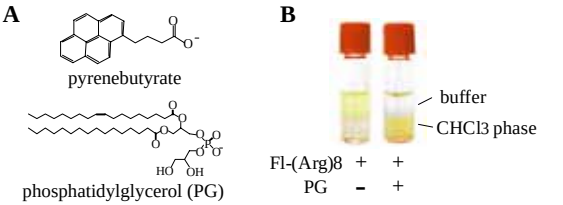


***Figure 1***. (**A**) Structures of two representatives of negatively charged counteranions with high hydrophobicity. (**B**) Typical results of extraction experiment with Fl-(Arg)8 (25 μM), PG (left, 0 mM;

## 2. Conteranion-mediated delivery of arginine-rich peptide into cytosol

The guanidinium cations in the above-mentioned vectors can bind tightly to negatively charged small molecules with high hydrophobicity (Figure 1A). We have shown that the counteranions can have a large effect on the behavior of arginine-rich peptides in the artificial membranes [2,3]. Figure 1B shows a typical extraction

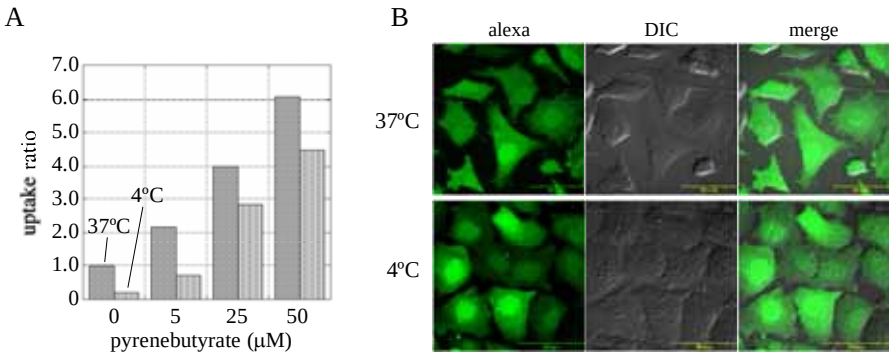


***Figure 2.*** (**A**) HeLa cells were pre-treated with pyrenebutyrate for 5 min prior to a 15-min incubation with fluorescently labeled R8 peptide (5 μM) in phosphate buffered saline (PBS), washing with cold PBS and analysis by flow cytometry. (**B**) HeLa cells were pre-incubated with pyrenebutyrate (50 μM) for 2 min prior to 4-min incubation with R8 peptide (10 μM) and analyzed by confocal microscopy.

experiment that hydrophilic octaarginine labeled with fluorescein [Fl-(Arg)8] can be transferred into hydrophobic environment ($CH_3Cl$) with the help of a counteranion (PG). It has been thought that the positively charged arginine-rich peptides electrostatically interact with the counteranions to increase their net hydrophobicity and therefore to directly translocate through the lipid bilayer. After confirming that this counteranion-mediated direct translocation of arginine-rich peptides occurs in artificial liposomes, we have investigated whether counteranions can have effects on the cellular uptake of octaarginine (R8), one of the representative arginine-rich peptides.

When HeLa cells were treated with R8 peptide in the presence of pyrenebutyrate, a representative counteranion, to analyze the total amount of the internalized R8 peptide using a flow cytometer, a gradual increase in uptake of R8 peptide was observed in accordance with increase in concentration of pyrenebutyrate (Figure 2A). In microscopic observation, a diffuse cytosolic localization of the internalized R8 peptide was achieved in a few minutes (Figure 2B). In addition, we found that these effects of pyrenebutyrate were significantly suppressed by cancellation of membrane potential. These facts suggest that the interaction with hydrophobic counteranions allows the hydrophilic arginine peptides to easily go through the hydrophobic biological membranes, and that the membrane potential would play critical roles in this process. Using this counteranion-mediated delivery, we have succeeded in delivery of a bioactive molecule and even large molecular weight protein into cytosol.

## 3. Conclusion

This counteranion-mediated approach enables us to achieve direct, efficient and reversible delivery of bioactive proteins bearing arginine vectors into the cytosol. The procedure is very simple with no detectable cytotoxicity. A few minutes' treatment of the cells with pyrenebutyrate/peptides in PBS is sufficient for the introduction. Considering these outstanding features, this concept of counteranion-mediated delivery using arginine-rich peptides should open new avenues for intracellular protein delivery and imagings of intracellular molecules.

## *References*

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*Emerging approaches for real-time molecular and cellular imaging*

***Speaker:***
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# VISUALIZATION AND MANIPULATION OF CELLULAR EVENTS USING FLUORESCENCE SENSOR MOLECULES

## 1. Introduction

One of the great challenges in the post-genome era is to clarify the biological significance of intracellular molecules directly in living cells. If we can visualize a molecule in action, it is possible to acquire biological information, which is unavailable if we deal with cell homogenates. One possible approach is to design and synthesize chemical probes that can convert biological information to chemical reactions that are easily monitored. For this purpose, fluorescent sensor molecules for intracellular messengers have been developed and successfully applied to living cells.

## 2. $Zn^{2+}$ Imaging

Recently, the physiological significance of chelatable $Zn^{2+}$ has attracted much attention. However little is known about the cellular regulation of $Zn^{2+}$, so several chemical tools for measuring $Zn^{2+}$ in living cells have been developed. Fluorescent sensor molecules for $Zn^{2+}$, ZnAF-1 and ZnAF-2, were developed based on photo-induced electron transfer (PET) and their experimental utility to detect $Zn^{2+}$ from living cells was examined (Fig). ZnAFs, whose acceptor for $Zn^{2+}$ *N,N*-bis(2-pyridylmethyl)ethylenediamine is directly attached to the fluorescein moiety via the aliphatic amine nitrogen, showed high sensitivity in nM range and low background fluorescence. The dyes were specific for $Zn^{2+}$ against other heavy metal

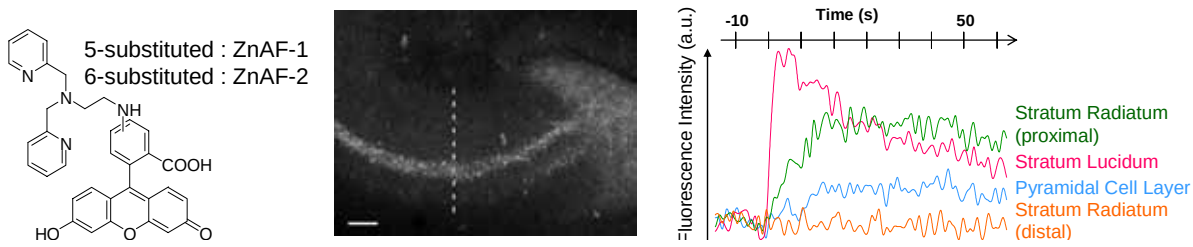


Figure. Structure of ZnAFs and Rat Hippocampal Imaging of $Zn^{2+}$ Release.

ions and other cations, that exist at high concentration in living cells such as $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ and $K^{+}$. Labile $Zn^{2+}$ was shown to play an important role in the central nerve system, where it is mainly stored in the synaptic vesicles of excitatory synapses, particularly the synaptic terminals of hippocampal mossy fibers. It was shown that $Zn^{2+}$ was co-released

with neurotransmitters in response to synaptic activity. Electrophysiological analyses revealed that NMDA-receptor-mediated synaptic responses ($fEPSP_{NMDA}$) in CA3 were inhibited by this $Zn^{2+}$ release, whicah indicates that $Zn^{2+}$ serves as a heterosynaptic mediator

### 3. Modulation of Lanthanide Luminescence Intensity by $Zn^{2+}$ Chelation

Lanthanide complex based $Zn^{2+}$ sensors were also developed. Lanthanide complexes ($Eu^{3+}$, $Tb^{3+}$, etc.) have excellent spectroscopic properties for biological applications, such as long luminescence lifetimes of the order of milliseconds, a large Stoke's shift of >200 nm and high water-solubility. This europium ($Eu^{3+}$) complex employs a quinolyl ligand as both a chromophore and an acceptor for $Zn^{2+}$. Upon addition of $Zn^{2+}$ to a solution of **[Eu-7]**, the luminescence of $Eu^{3+}$ is strongly enhanced, with high selectivity for $Zn^{2+}$ over other biologically relevant metal ions. The usefulness of **[Eu-7]** for monitoring of $Zn^{2+}$ changes in living HeLa cells was shown using fluorescence microscope with time-resolved fluorescence (TRF) measurement equipment. The exclusion of background fluorescence using **[Eu-7]** and TRF gave much clearer images of $Zn^{2+}$ signal in living cells than images taken without TRF method.

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