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研究課題名（和文）電子顕微鏡観察のためのSNAPタグ技術の開発

研究課題名（英文）Development of SNAP-tag technology for deep-etch electron microscopy

研究代表者

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研究成果の概要（和文）：まず、SNAPタグ-β-2アドレナリン受容体タンパク質をプラスミドコードを用いて、最適な電子顕微鏡（EM）の細胞のラベリングのための条件を確立しました。そして、SNAPタグ方式を適用しました。モデルとして、カベオリン1タンパク質を使用しました。まず、正常にSNAP-タグプラスミドにカベオリン1遺伝子をクローニングし、その後、細胞を共焦点顕微鏡を用いて、カベオラの特徴が見つけることができました。次に、SNAPタグシステムを分析しました。まず急速凍結および凍結乾燥細胞を、カベオラにおけるカベオリン-1の特異的標識を確認するために、EMによりそれらを観察しました。

研究成果の概要（英文）：First, we established the conditions for an optimal electron microscopy (EM) cellular labeling by using a plasmid encoding for SNAP tag-β-2 adrenergic receptor protein. Then, we applied SNAP tag method to achieve the EM labeling for basal membrane sheets and intracellular proteins. We have used Caveolin-1 protein, as a model, as it has been widely studied in our lab. First, we successfully cloned Caveolin-1 gene into the SNAP-tag plasmid. Then we checked it by confocal microscopy, both with live and fixed cells and there was a nice punctuated labeling, characteristic of caveolae. Thus, we assayed the SNAP tag system for the labeling of basal membrane sheets. After quick-freezing and freeze-drying the cells, we observed them by TEM to confirm the specific labeling of Caveolin-1 in caveolae. We had to find the optimal conditions for the labeling of unroofed cells. The most difficult part was to find a good nanoparticle-streptavidin conjugate with electron microscope quality.

研究分野：　生物学

キーワード：　Bioimaging　Electron microscopy

１．研究開始当初の背景

Electron microscopy (EM) is a powerful imaging technique, its high spatial resolution (in the order of few nanometers) allows for a detailed observation of cellular structures.
However, visualizing specific molecules by EM while maintaining structural details, needed for the understanding of molecular and cellular functions, remains problematic. In other words, while everything in the cell can be seen (all of the organelles, and membranes), it is still a challenge to simultaneously visualize molecules of interest.
On the contrary, by light microscopy, molecules of interest are routinely labelled by a fluorescent tag to monitor their dynamics and cellular localizations, however, cellular structures cannot be visualized.
If molecules could be labelled and localized in electron microscopy as easily as in light microscopy, it would be a huge advancement for the field of cellular biology.
There have been some attempts to do so, but none of them is easy to perform and the results are not as clear and fast to interpret as those using light microscopy.
One of the recent technologies successfully developed for light microscopy is SNAP-tag technology. The SNAP-tag method consists of expressing the protein of interest fused with a SNAP-tag which reacts with benzylguanine (BG) derivatives.
Moreover, SNAP-tag can be used in combination with a similar tag, termed CLIP-tag, therefore allowing for the simultaneous labelling of two different proteins.
Until now, SNAP-tag has only been used for light microscopy purposes, but we believe that it can be a wonderful tool for electron microscopy as well. Not only can SNAP-tag technology be used in fixed cells (thus overcoming the problems of immunogold-based methods), but it also shows great specificity and allows for light microscopy without cytotoxicity issues.
In the proposed project, we will develop SNAP-tag technology for electron microscopy by preparing BG derivatives with electrodense probes (gold nanoparticles, quantum dots) and proving that it will work for EM, initially with unroofed cells, and afterwards with whole cells.

２．研究の目的

The objective of this work is to develop a new, easy to perform and rapid interpretable method for labelling specific molecules for EM.
Being able to work with fixed cells significantly improves the ultrastructural quality of the samples. Also, the higher labelling efficiency of SNAP-tag compared with antibodies reveals a much more detailed distribution of the proteins inside the cells. This will help in the elucidation of the molecular basis of several cellular processes, like trafficking, cell division, cell growth or cellular differentiation.
Furthermore, ease of sample preparation using the SNAP-tag method allows different types of cell preparations, such as thin sections, unroofed, freeze-fractured and freeze-dried cells.
Moreover, the SNAP-tag method is potentially applicable to all kinds of proteins, overcoming the problems of DAB based methods (only valid for protein assemblies) or immunogold-based methods (where steric hindrance limits the ability of proteins to be labelled).
When working with quantum dots as a probe, correlative light electron microscopy will be possible. It is important to note that unlike DAB based genetically encoded methods, where cells have to be fixed due to cytotoxicity problems, SNAP-tag method will allow for live cell imaging. This will combine the power of LM to observe the dynamics of cellular processes with the resolution of EM for ultrastructural determination.

３．研究の方法

SNAP tag biotechnology:

The SNAP-tag method consists of expressing the protein of interest fused with a SNAP-tag, a 20 kDa mutant of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase, which reacts specifically and quickly with benzylguanine (BG) derivatives.
The reaction of SNAP-tag with BG is covalent and irreversible, and the reaction yield seems to be to a large extent independent of the probe attached to BG. Importantly, SNAP-tag substrates are very specific and unreactive with other proteins, thus avoiding nonspecific labelling.

Quick-Freeze, Deep-etch electron microscopy:

The first step of EM sample preparation is quick-freezing. Samples are being freezed ultra-rapidly by 'slamming' them against a cold copper block, which is being cooled down all the way to liquid helium temperature (-269 °C). This ultra rapid freezing improves the ultrastructural quality of the sample (smaller ice crystals are formed).
Also it is remarkable to point out that live cells can be cryo-fixed using this method.
Then the sample is introduced to the freeze fracture machine. If the cell interior has to be observed then one has to proceed to do freeze-fracture. In the case of wanting to observe the whole cell, freeze-drying procedure is taken.
After the etching process they are platinum and carbon sprayed as a thin platinum replica can be viewed using the transmission EM.
Platinum replicas give exceedingly high contrast and are almost insensible to electron-beam damage.
This platinum and carbon replica is then observed at the transmission electron microscope (TEM).

４．研究成果

We have started taking the first steps towards demonstrating that SNAP-tag technology can be a wonderful tool for electron microscopy:
Before doing the cloning for Caveolin-1 protein we wanted to establish the conditions for an optimal electron microscopy cellular labeling, and we did so using an already existing plasmid, encoding for SNAP tag-β-2 adrenergic receptor protein.
Using this system we could probe the ease of sample preparation, finding the optimal conditions for the labeling of whole cells, like incubation time, concentration of the BG gold nanoparticle probe, etc.
After quick-freezing and freeze-drying the cells we conducted the observation by transmission electron microscopy (TEM) and we could see a beautiful labeling of gold nanoparticles on the surface of freeze-dried cells.
During the second year we have applied SNAP tag method to achieve the electron microscopy labeling for basal membrane sheets and intracellular proteins. We have used Caveolin-1 protein, as a model, as it has been widely studied in our lab.
First, we successfully cloned Caveolin-1 gene into the SNAP-tag plasmid. Then we checked it by confocal microscopy (CLSM), both with live and fixed cells. For those experiments we used either BG-OregonGreen or BG-Biotin, with a further step of Streptavidin-AF488. In the first case we could observe the cells either live or fixed. In the case of using the biotin-streptavidin we had to work with fixed and permeabilised cells or with unroofed cells. We checked the cells by CLSM and there was a nice punctuated labeling, characteristic of caveolae.
The labeling worked both for whole cells (Figure 1) or unroofed cells (Figure 2).

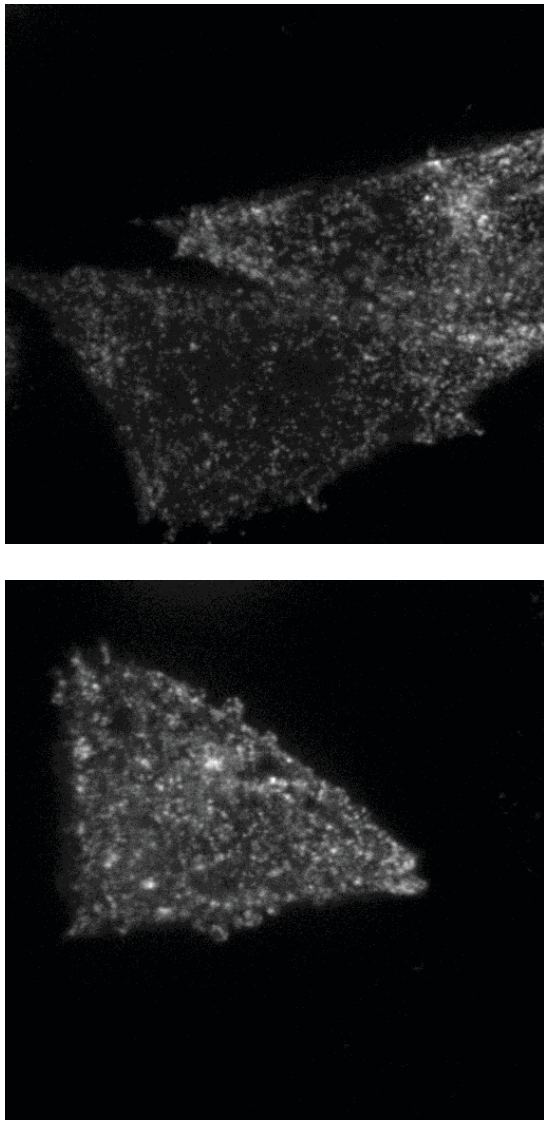
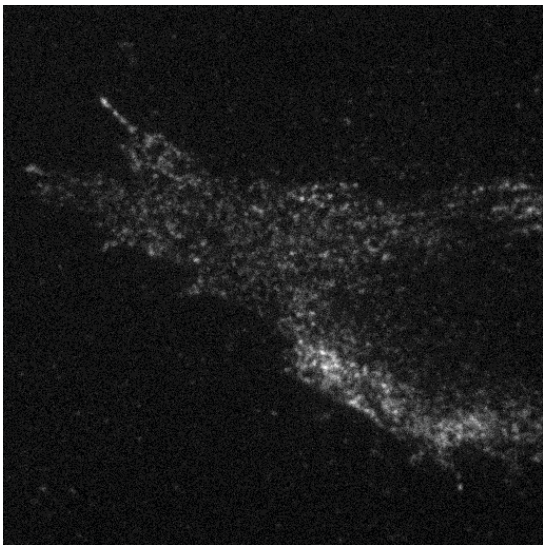


Figure 1. T24 cells expressing Caveolin 1-SNAP tag labeled with BG-Oregon Green. Whole cell, BG labeling done after fixation.



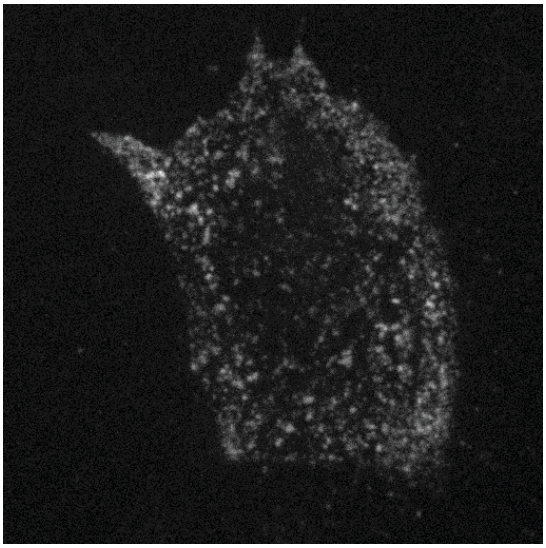
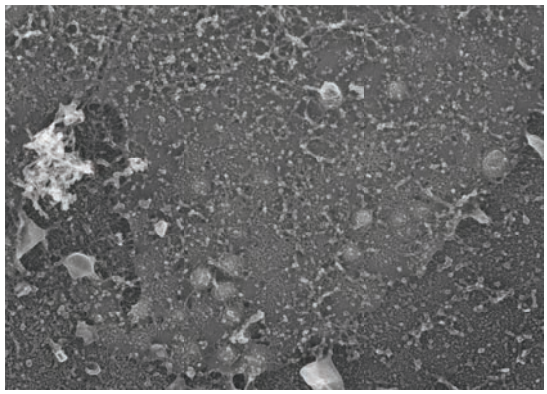


Figure 2. T24 cells expressing Caveolin 1-SNAP tag labeled with BG-Oregon Green. Unroofed cell, BG labeling done after fixation.

We took the next steps and performed electron microscopy (EM). The EM technique that we use is called quick freeze, deep etch EM, which is able to freeze cells instantly and preserve the cellular structures intact. Then after etching the sample and rotatory evaporating platinum and C over it, it is ready for transmission EM (TEM) observation.
Thus, we assayed the SNAP tag system for the labeling of basal membrane sheets. After quick-freezing and freeze-drying the cells, we observed them by TEM to confirm the specific labeling of Caveolin-1 in caveolae. We had to find the optimal conditions for the labeling of unroofed cells (incubation time, concentration of BG-biotin, nanoparticle-streptavidin, etc.). The most difficult part was to find a good nanoparticle-streptavidin conjugate with electron microscope quality.
The most relevant aspect is that being able to work with fixed cells has significantly improved the ultrastructural quality of the sample.



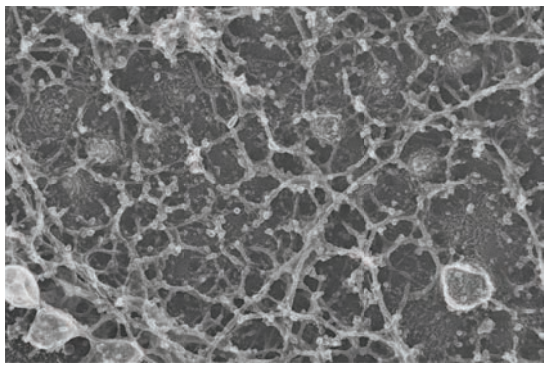


Figure 3. TEM micrograph: T24 unroofed cell expressing Caveolin 1-SNAP tag labeled with BG-Biotin/Streptavidin gold.

５．主な発表論文等

〔学会発表〕（計　1　件）

Pujals, S., "Workshop on 3D solutions in Cryo-Electron Microscopy 2015", July 6-10, 2015, M4I Division of Nanoscopy, Maastricht University, The Netherlands. Short oral communication: “Quick-freeze, Deep-etch Electron
Microscopy”

６．研究組織

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