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Effects of employment of distinct strategies to capture antibody on antibody delivery into cultured cells

Kana Kuwahara,[1,2] Kazuki Harada,[1,2] Ryohei Yamagoshi,[1,2] Takenori Yamamoto,[1,2] and Yasuo Shinohara[1,2],*

[1] Institute for Genome Research, University of Tokushima, Kuramoto-cho-3, Tokushima 770-8503, Japan

[2] Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi-1, Tokushima 770-8505, Japan

Footnotes:

* To whom correspondence should be addressed: Yasuo Shinohara, Institute for Genome Research, University of Tokushima, Kuramoto-cho-3, Tokushima 770-8503, Japan, Phone: +81-88-633-9145, Fax: +81-88-633-9146, E-mail: yshinoha@genome.tokushima-u.ac.jp

ABSTRACT

The characteristics of antibody delivery into cultured HeLa cells were examined by using two delivery systems. Both systems used a cell-penetrating peptide as a tool for intrusion of an antibody into the cells, but either a "protein A derivative" or "hydrophobic motif" was employed to capture the antibody. When we examined the uptake of the Alexa Fluor-labeled antibody by use of these two systems, both systems were found to effectively deliver the antibody into the cultured cells. However, when we compared the amount of antibody delivered by these systems with the amount of transferrin uptake, the former was 10 times smaller than the latter. The lower efficiency of antibody delivery than transferrin uptake seemed to be attributable to the involvement of the antibody delivery reagent, which failed to catch the antibody molecule. This interpretation was validated by an experiment using a larger amount of antibody, and the amount of antibody delivered by the "protein A derivative" system under this condition was determined to be 13 ng proteins/10^5 cells. The antibody delivery achieved by the "protein A derivative" or "hydrophobic motif" showed two differences, i.e., a difference in intracellular distribution of the delivered antibody molecules and a difference in the fluorescence spectrum observed with cellular lysates. Possible reasons for these differences between the two delivery systems are discussed.

Keywords: antibody delivery, cell-penetrating peptide, protein A, hydrophobic motif

INTRODUCTION

In the field of life sciences, antibodies are powerful experimental tools used for specific detection of certain proteins or chemicals, or for measurement of their amounts. Nowadays, this tool is also widely used for therapeutic purposes against diseases such as autoimmunity, inflammation or cancer (for reviews, see [1,2]). The major targets of antibody in the latter clinical applications are relatively limited to be extracellular water-soluble proteins such as cytokines or membrane proteins present on the cell surface, and antibody therapy targeting subcellular proteins has never been established. To explore the possible application of antibody for artificial regulation of intracellular biological processes, it is obviously essential to deliver the antibody into the living cell. Recently, trials to develop tools useful for delivery of extracellular proteins into living cells have been made, and some of them have become commercially available. These tools must be capable of i) allowing entry of the protein into the cells and ii) capturing the target protein molecule to be delivered into the cells. The major strategies used in these systems for getting a protein into living cells are the use of i) cationic lipids as a carrier [3,4] or ii) a cell-penetrating peptide [5-7]. As for the strategies to capture the target protein, non-specific interactions such as electrostatic or hydrophobic interactions are employed in most systems [3-6]. The use of non-specific interactions as a strategy to capture the target protein molecule has an advantage in that it could be applicable for various protein species. However, possible disadvantages of the use of such non-specific interactions as a strategy are uncertain. Recently, an antibody delivery tool using a cell-penetrating peptide and a derivative of protein A was developed [8-10]. As we considered it of interest to examine the effects of the usage of different strategies to capture an antibody for its delivery into cells, in the present study we compared the utility of two systems. Both systems employed a cell-penetrating peptide as a tool to deliver the antibody into living cells, but they used either a protein A derivative or non-specific interactions as a strategy to capture the antibody molecules (for structural properties of these two systems employed in the present study, see **Fig. 1**).

MATERIALS and METHODS

Materials -

HeLa cells were obtained from JCRB (Japanese Collection of Research Bioresources) Cell Bank.

Eagle's minimum essential medium (code 5900) was purchased from Nissui (Tokyo). Non-essential amino acids (code 11140-019), goat anti-human IgG, Alexa Fluor 488 conjugate (code A11013), transferrin from human serum, and Alexa Fluor 488 conjugate (code T13342) were obtained from Life Technologies, Japan (Tokyo).

Chariot (code 30025), a cell-penetrating protein delivery agent, and Ab-Carrier (code P-101-25), a cell-penetrating antibody delivery agent, were purchased from Active Motif (Carlsbad) and ProteNova (Naruto, Japan), respectively.

Cell culture and antibody transfection studies –

HeLa cells were cultured in MEM (Eagle's minimum essential medium) supplemented with non-essential amino acids (0.1 mM each) and 10% fetal bovine serum [11].

For antibody transfection studies, $2 \sim 3 \times 10^5$ HeLa cells were plated in culture dishes (35-mm diameter, Iwaki code 3810-006); and after 24 hrs' incubation, the cells were washed twice with PBS(-) medium, and then subsequently treated with the individual transfection systems. Detailed experimental protocols used for individual antibody transfection are depicted in **Supplementary Fig. S1**.

Fluorescence imaging of the protein/antibody delivered into cells -

For observation of the fluorophore-conjugated protein/antibody, the cells treated with individual transfection systems were washed for 3 times with acidic glycine buffer (200 mM glycine, pH 3.0, containing 150 mM NaCl) to eliminate non-specifically attached molecules [12], and then once with PBS(-) medium.

Uptake of transferrin or antibody conjugated with fluorescence dye was monitored by using a fluorescence microscope (EVOS, model AMF-4301), with LED fluorescence cube "GFP" (wavelengths used for excitation and emission of 470 and 525 nm, respectively, and parameters for gain, brightness, and contrast of 100%, 72%, and 72%, respectively).

Preparation of cellular lysates and determination of fluorescence spectrum -

Cells in 35-mm dishes were intensively washed as described above, and then detached from the culture dishes by adding 40 mM Tris-Cl buffer, pH 7.5, containing 150 mM

NaCl and 1 mM EDTA . The resulting cell suspension was centrifuged at 15,000 rpm, 4° C for 5 min; and after removal of the supernatant, the cells were lysed by adding 200 μl of lysis buffer (20 mM Tris-Cl buffer, pH 8.0, containing 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 10% glycerol, 0.5% Triton X-100, and 1% Nonidet P-40). After removal of non-dissolved residues by centrifugation, the fluorescence spectrum of the supernatant was determined by using an INFINITE microplate reader, model F200 PRO (TECAN) with excitation at 480 nm.

RESULTS and DISCUSSION

In the present study, we employed a protein delivery reagent and an antibody delivery reagent. Both reagents use a cell-penetrating peptide as a strategy to deliver a protein or antibody into cells [5,10]; but the former and the latter reagents used a "hydrophobic motif" and "protein A derivative" to capture a target protein and antibody molecule, respectively (**Fig. 1**). In this report, the former and latter reagents will be simply referred to as delivery systems using a "hydrophobic motif" and "protein A", respectively.

First of all, we examined whether these systems would be actually effective in the delivery of antibody into cultured cells. For this, fluorophore-conjugated antibody was mixed with a "hydrophobic motif" or with "protein A", and HeLa cells were treated with one of these mixtures (for detailed experimental conditions, see **Supplementary Fig. S1**).

Uptake of transferrin, which is well established to be achieved *via* a receptor-mediated endocytotic pathway [13], was used as a positive control of protein uptake into cultured HeLa cells. Because the standard experimental protocol of the protein A system recommended by the supplier employs 4 μg of antibody per well, to enable quantitative comparison of the efficiencies of the protein delivery by the two delivery systems and that of transferrin uptake, we performed default experiments using 4 μg of antibody and 4 μg of transferrin.

As shown in **Fig. 2**, upper panel, when cells were incubated only with antibody molecules having a fluorophore (i.e., negative control), no cells showed fluorescence, indicating that the cells did not spontaneously take up the antibody. However, when the cells were incubated with antibody molecules complexed with "protein A" or with the "hydrophobic motif", they showed clear fluorescence in their interior, indicating that these reagents were effective in the delivery of antibody into the cultured cells. Under this condition, no remarkable difference in antibody uptake was observed between the two systems.

Although the protein species are completely different (i.e., antibody *vs*. transferrin), we could roughly compare the efficiencies of protein uptake by comparing the signal intensity of fluorescence in the cells incubated with the transferrin with that of the antibody molecules, because the degree of fluorophore labeling of the individual proteins (molecules per gram of protein) was almost the same between antibody and transferrin (stated in the datasheets of the products). It should be noted that the molecular size of transferrin (approx. 80 kDa) is about half of that of antibody (in the case of IgG, approx. 150 kDa).

When we compared the results obtained for transferrin uptake with those for uptake of antibody complexed with "protein A" or with the "hydrophobic motif" the difference in the signal intensity between transferrin and antibody was remarkable; i.e., the signal intensity of the former (**Fig. 2**, upper panel, "+ Transferrin (4 μg)") was extremely stronger than that of the cells incubated with antibodies, even though the cells had been incubated with identical amounts (4 μg) of transferrin and antibody. To enable more precise comparison of the signal intensity, we incubated cells with various amounts of transferrin, and then evaluated by fluorescence microscopy the signal intensity of the transferrin taken up. As shown in **Fig. 2**, lower panel, the intensity of fluorescence of transferrin taken up by the HeLa cells changed in a dose-dependent manner with respect to transferrin; and cells incubated with 0.4 μg of transferrin showed a signal intensity similar to that of cells incubated with 4 μg of antibody complexed with "protein A" or with "hydrophobic motif" (see also **Fig. 3**, upper panel). These results seemed to indicate that the efficiency of antibody delivery by "protein A" or with the "hydrophobic motif" was about ~10% of the efficiency of transferrin uptake.

We assumed that the reason for this difference could be attributable to failure of the delivery reagent to capture the antibody molecule. If this interpretation is correct, an increase in the amount of antibody subjected to be complexed with "protein A" or with "hydrophobic motif" would be expected to cause elevated uptake of the antibody. As shown in **Fig. 3**, lower panel, when we used 20 μg of antibody, the signal intensity of the fluorescence taken up by the cells increased several folds.

Under this condition, moreover, it was evident that the distribution pattern of the fluorescence in the cells was distinct between "protein A" and "hydrophobic motif". That is, the fluorescence was distributed relatively homogeneously in the cells treated with antibody mixed with "protein A", but was observed in just certain regions of the cells treated with antibody mixed with the hydrophobic motif. This latter distribution pattern was also observed with transferrin. At this moment, the reason for this difference in distribution is uncertain.

In the above studies, we roughly evaluated the uptake of antibody by comparing the signal intensity of the fluorescence observed by fluorescence microscopy. To enable more quantitative evaluation of the antibody uptake, we next prepared cellular lysates and measured their fluorescence spectrum. For this purpose, cells were incubated with antibodies as shown in **Fig. 3**, lower panel (i.e., cells were incubated with 2 μg of transferrin or with 20 μg of antibody complexed with protein A or hydrophobic motif), after which their lysates were prepared.

As shown in **Fig. 4**, the lysate of the cells incubated with transferrin or of the cells

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treated with antibody complexed with "protein A" (green and red traces, respectively, in the left panel) showed essentially the same fluorescence spectrum (λmax ~**525** nm) as that observed with the diluted solution of Alexa-labeled transferrin or antibody (traces in the right panel). When we calculated the amount of transferrin or antibody taken up by the HeLa cells, it was determined to be ~ 13 ng proteins/10^5 cells. On the contrary, the lysate of cells treated with antibody complexed with the "hydrophobic motif" showed a red-shift in the fluorescence spectrum (**Fig. 4**, blue trace in the left panel, λmax ~565 nm); and because of this shift, accurate calculation of the amount of antibodies taken up by the cells was difficult. As this red-shift in the fluorescence spectrum was suspected to be attributable to the results of non-specific interactions between antibody and the "hydrophobic motif" we examined whether a red-shift in the fluorescence spectrum of an antibody solution would be caused by addition of the "hydrophobic motif". However, at least under the conditions used, no red-shift in the fluorescence spectrum caused by the addition of the "hydrophobic motif" was observed (**Fig. 4,** blue-dotted trace in the left panel). Thus, the reasons for this red-shift in the fluorescence spectrum remain elusive.

Because delivery of a certain protein(s) into cultured cells has a great impact on the artificial control of living cells, studies on the delivery tools have been intensively performed. As a result, the use of cationic lipids or cell-penetrating peptide was found to be an effective strategy for the penetration of macromolecules into cultured cells [3-7]; and several delivery systems have been commercialized. Recent studies have mainly focused on the molecular mechanisms of internalization of proteins into cultured cells [14,15] or on the escape from endosomes [16,17].

The protein delivery system has also started to be employed for antibody delivery, especially to examine the effects of down-regulation of the target protein [18-20]. In addition to the protein delivery system, delivery systems specific for antibody have also been developed [8-10]; but the question as to how they differ has not yet been answered. Thus, in the present study, we compared the utility of two systems: both systems employed a cell-penetrating peptide as a tool for entry of antibody into living cells, but they used either a protein A derivative or non-specific interactions as a strategy to capture the antibody molecules. In the case of the antibody delivery, evaluation of the amount of the antibody delivered into the cells is very important. Therefore, we characterized the properties of antibody delivery by these two delivery systems in a quantitative manner, and arrived at the following conclusions: i) both reagents were effective in delivering the antibody molecules into cultured cells, but the amount of delivered antibody was 10 times lower than the amount of transferrin taken up by the

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cultured cells, ii) when cells were treated with 4 μg of antibody complexed with "protein A", 13 ng of antibody could be taken up by 10^5 cells, and iii) the antibody delivery achieved by the "protein A" or "hydrophobic motif" showed two differences, i.e., a difference in intracellular distribution of the delivered antibody molecules and a difference in the fluorescence spectrum observed with cellular lysates.

As for the amounts of antibody delivered into cells, Mussbach *et al*. reported the value of 4.3 amol/cell [21]. Although they employed an experimental technique completely distinct from ours, i.e., they quantified the antibody by measuring the fluorescence of the FITC-labeled antibody after separation by SDS-PAGE; and the obtained value was relatively similar to our value (just 5-times higher than ours). These results indicate that these values are highly reliable. However, further discussion on the possible reasons for the observed difference would be difficult, because the detailed features of the delivery system they employed are uncertain. As for the difference in distribution of fluorescence between cells treated with "protein A" or the "hydrophobic motif", the pathway used for the antibody uptake may have the most significant influence. The two antibody delivery systems used in the present study employed "cell-penetrating peptide" for entry of the antibody into the cells; but in an exact sense, the structure of the peptide used was different between these two systems [5,10]. To obtain a clear explanation for this difference, comparison of the systems having a completely identical cell-penetrating peptide would be required. To understand the reasons causing the spectrum shift of Alexa Fluor observed with the lysate of the cells treated with antibody complexed with the "hydrophobic motif", further studies on the interaction between delivery reagents and antibody, and on the effects of different subcellular distributions on the fluorescence Alexa Fluor, will be necessary.

Very recently, Marschall *et al*. reported some important results regarding antibody delivery [22]. They developed a sophisticated method for evaluation of the amount of antibody present in the cytosol by using the Cre/LoxP recombinant system, and reported that the amount of "functional" antibody successfully delivered into the cytosol is very small. Because artificial regulation of cellular functions by antibodies would seem to have high potential, further approaches from various aspects seem necessary.

Acknowledgements -

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LEGENDS for FIGURES

Fig. 1. Structural properties of the two protein delivery systems used in the present study

In the case of protein (or antibody) delivery systems using a cell-penetrating peptide, the regions (or domains) responsible for their two functions of "entry into cells" and "capture of target protein" are separately shown. In the present study, we evaluated the antibody delivery achieved by these two delivery systems, one having a "hydrophobic motif" and the other, a "protein A derivative".

Fig. 2. Observations of the cellular uptake of transferrin or antibodies by fluorescence microscopy

Antibody delivery achieved by the two delivery systems and the cellular uptake of transferrin were evaluated by fluorescence microscopic analysis. Upper photos: cells were incubated with Alexa-labeled antibody (4 μg) complexed with either "protein A" or "hydrophobic motif" (right two photos). The result obtained with naked Alexa-labeled antibody is also shown as a negative control (center photo). The photographs of the non-treated cells and cells incubated with 4 μg of transferrin are also shown (left two photos). Lower photos show the dose-dependent uptake of transferrin. For detailed experimental conditions, see the Methods section and **Supplementary Fig. S1**.

Fig. 3. Effects of the amounts of antibody on the antibody delivery through "protein A" or "hydrophobic motif"

The upper panel shows the results obtained with the cells incubated with 0.4 μg of transferrin, or incubated with 4 μg of antibody complexed either with "protein A" or the "hydrophobic motif". The lower panel gives the results obtained from the experiment using larger amounts of transferrin (2 μg) or antibody (20 μg). Note that the amounts of protein A and hydrophobic motif used in the upper and lower panels are identical.

Fig. 4. Fluorescence spectrum observed with the lysates of cells incubated with transferrin or with antibody complexed with "protein A" or with the "hydrophobic motif"

The solid traces in the left panel show the fluorescence spectrum obtained for the lysates of cells incubated with transferrin (green), with naked antibody (yellow), with antibody complexed with "protein A" (red) or with antibody complexed with the "hydrophobic

motif” (blue). The cellular lysates were prepared from the cells treated as stated in Fig. 3, lower panel. The dotted blue trace in the left panel indicates the fluorescence spectrum of antibody solution just mixed with the “hydrophobic motif”. The right panel gives the fluorescence spectrum of the aqueous solution of transferrin and antibody as controls (calibration).

LEGEND for SUPPLEMENTARY FIGURE

Supplementary Fig. S1. Detailed experimental procedures for the antibody-uptake experiments

The antibody molecule conjugated with Alexa Fluor is depicted as green symbol of Y-character shape. The delivery reagents of “protein A” and “hydrophobic motif” are depicted as schematically illustrated in Fig. 1.

Figure 1

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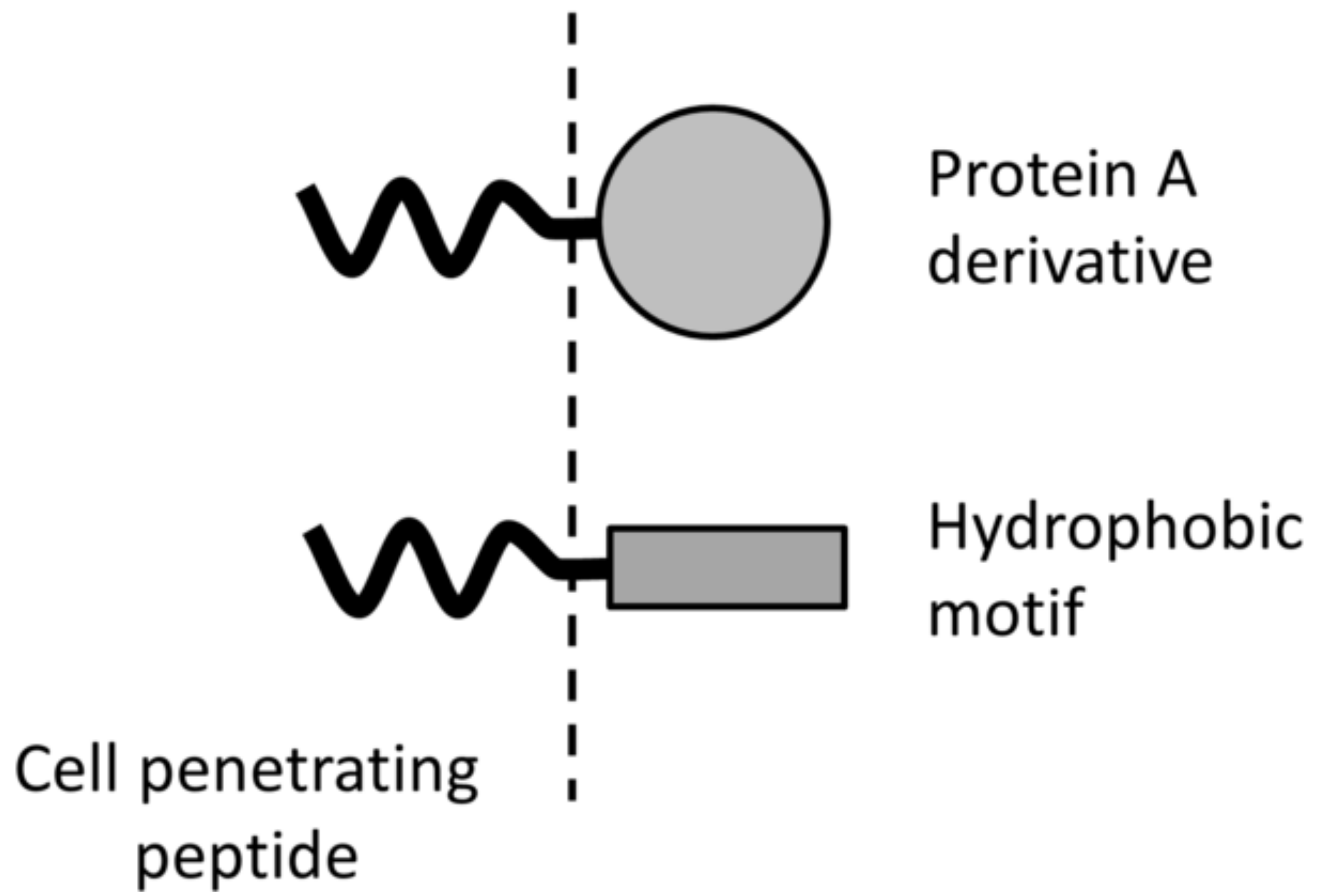

Figure 2

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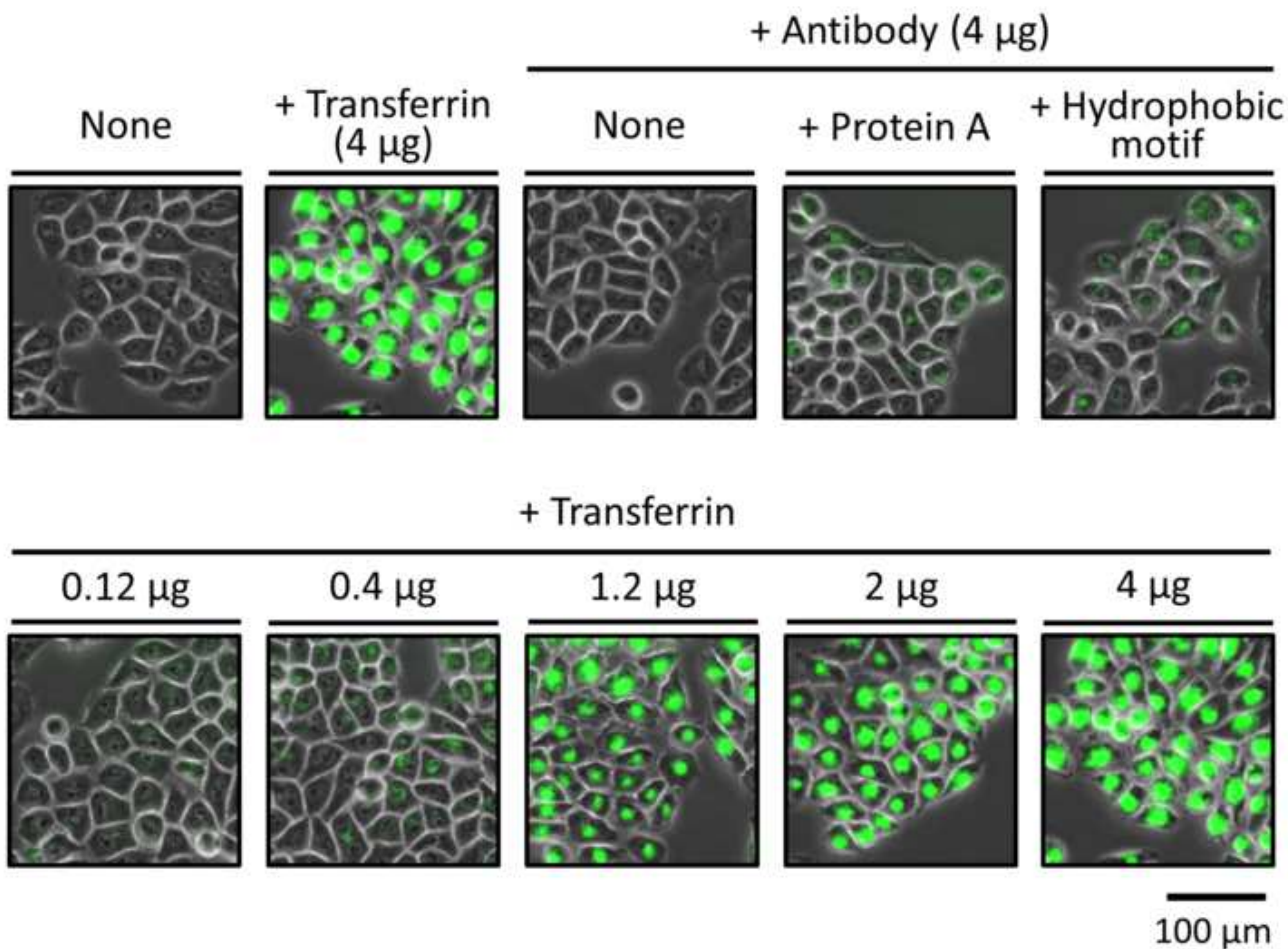

Figure 3
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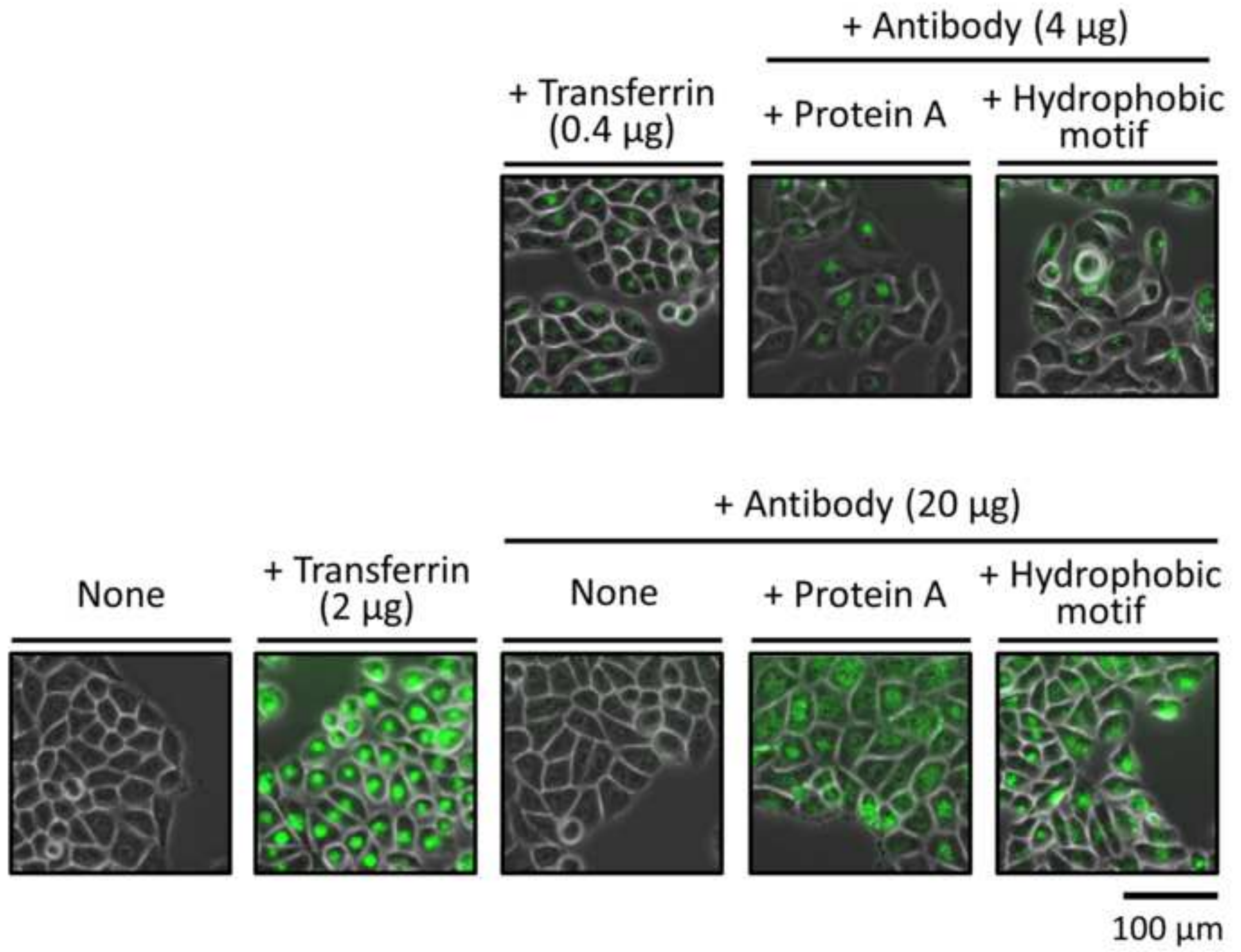

Figure 4

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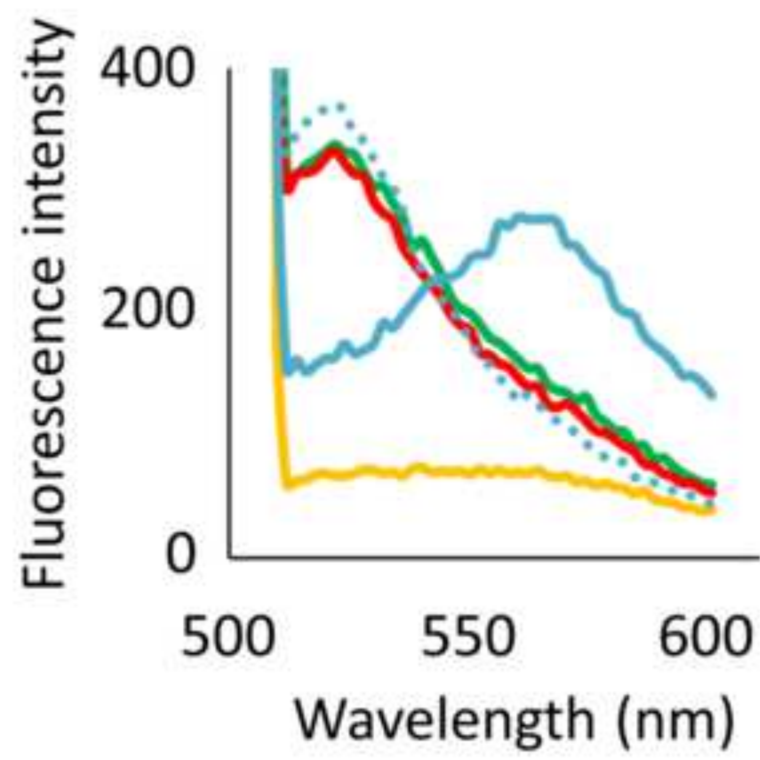
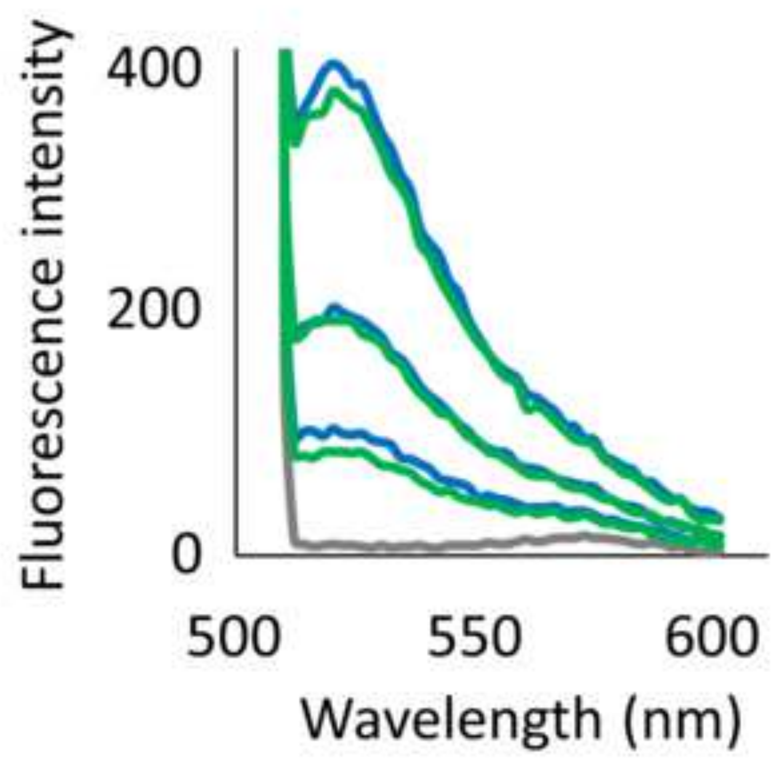




Transferrin

Antibody

Antibody + Protein A

Antibody + Hydrophobic motif

Antibody + Hydrophobic motif (in the absence of cells)

Buffer

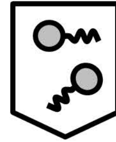
Transferrin

Antibody

(50, 100, 200 pg/μl)

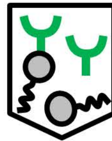
Protein A

antibody : 2 μL (4 μg)
1 × PBS(-) : 38 μL



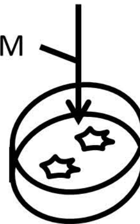
protein A : 2 μL
(approx. 0.64 μg)

mix together
incubate (20 min, RT)



protein A complex : 42 μL

1.96 mL MEM
(+FCS)



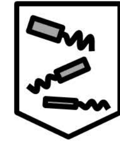
HeLa cells
30% confluent

total 2 mL

incubate
(4 hr, 37°C, 5% CO_2)

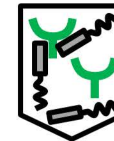
Hydrophobic motif

antibody : 2 μL (4 μg)
1 × PBS(-) : 98 μL



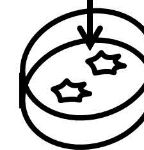
motif : 6 μL (12 μg)
water : 94 μL

mix together
incubate (30 min, RT)



Hydrophobic motif complex : 200 μL

0.4 mL MEM
(- FCS)



HeLa cells
40% confluent

total 0.6 mL

incubate
(1 hr, 37°C, 5% CO_2)

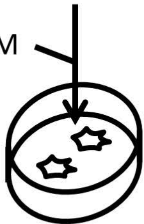
1.4 mL MEM
(+FCS)

incubate
(2 hr, 37°C, 5% CO_2)

Transferrin

transferrin : 20 μL (0.12 ~ 4 μg)

0.98 mL MEM
(+FCS)



HeLa cells
30% confluent

total 1 mL

incubate
(10 min, 37°C, 5% CO_2)

Fluorescence imaging