Rapid functional analysis of protein–protein interactions by fluorescent C-terminal labeling and single-molecule imaging

Junichi Yamaguchi[a], Naoto Nemoto[b,*], Toru Sasaki[b], Ako Tokumasu[b], Yuko Mimori-Kiyosue[c,1], Toshiki Yagi[d], Takashi Funatsu[a,e]

[a] *Department of Physics, School of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan*
[b] *GenCom Corporation, 11 Minamiooya, Machida, Tokyo 194-8511, Japan*
[c] *Tsukita Cell Axis Project, Exploratory Research for Advanced Technology, Japan Science and Technology Corporation, Kyoto Research Park, Shimogyo-ku, Kyoto 600-8813, Japan*
[d] *Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[e] *Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, Kawaguchi Center Building 1-8, Honcho 4-Chome, Kawaguchi-city, Saitama 332-0012, Japan*

Received 2 May 2001; revised 27 May 2001; accepted 28 May 2001

First published online 16 July 2001

Edited by Gianni Cesareni

Abstract Detection of protein–protein interactions is a fundamental step to understanding gene function. Here we report a sensitive and rapid method for assaying protein–protein interactions at the single-molecule level. Protein molecules were synthesized in a cell-free translation system in the presence of Cy5-puro, a fluorescent puromycin, using mRNA without a stop codon. The interaction of proteins thus prepared was visualized using a single-molecule imaging technique. As a demonstration of this method, a motor protein, kinesin, was labeled with Cy5-puro at an efficiency of about 90%, and the processive movement of kinesin along microtubules was observed by using total internal reflection microscopy. It took only 2 h from the synthesis of proteins to the functional analysis. This method is applicable to the functional analysis of various kinds of proteins.

Key words: Protein–protein interaction; Single-molecule imaging; Protein labeling; Puromycin; Fluorescence microscopy; Cell-free translation

1. Introduction

The draft sequence of the human genome has revealed a huge number of genes to be characterized [1,2]. The next challenge is to solve the human proteome to identify the functions of many different genes. The simplest and most direct method for analyzing the functions of the genes is to find the targets for the proteins encoded by these genes. To examine protein–protein interactions, two-hybrid systems, fluorescence resonance energy transfer, and conventional biochemical analysis have been employed to reveal hundreds of networks of protein interactions [3]. Single-molecule imaging using fluorescence microscopy has recently been developed. This method can be used to visualize individual interactions and to obtain invaluable information that is not extractable from conventional ensemble-averaged analysis [4–7]. Single-molecule imaging will be one of the most useful techniques for analyzing functions of unknown genes.

To make use of the power of single-molecule imaging, it is required to introduce a fluorescent molecule into a particular site of a protein without modifying its function easily and rapidly. Currently, no such method is available. Until now, the most general method for labeling a protein is chemical modification of the reactive cysteine that is substituted for an amino acid at a directed site [8]. However, there are several problems in this method. First, the selective labeling is difficult, particularly when the protein contains intrinsic cysteine residues. Secondly, proteins sometimes lose function as a result of the chemical modification. Thirdly, the standard chemical modification requires time and costs for genetic mutagenesis, expression and subsequent purification of the target protein. The last problem is a serious consideration in the post-genomic era when functional analysis should be done for a large number of proteins. As an alternative to chemical modification, green fluorescent protein (GFP) has been used as a fluorescent tag for target proteins [9,10]. It has the advantage that the labeling is possible both in vitro and in vivo [11]. However the function of the fusion protein is often inhibited due to large size of GFP (238 amino acids), so this method has serious limitations for the functional analyses of unknown proteins.

A promising candidate that replaces these methods is C-terminal labeling of proteins with fluorescent analog of puromycin [12]. This method is based on the fact that puromycin can be covalently attached to the C-terminal of proteins when a mRNA lacking a stop codon is synthesized in a cell-free translation system in the presence of puromycin at lower concentrations. Puromycin minimizes the influence of labeling on protein functions because of its small size. Although the concentration of the fluorescent protein obtained in the cell-free translation system is only several hundreds nanomolar, single-molecule assays can be performed in this low concentration of proteins. We expect that the combination of single-molecule

*Corresponding author. Fax: (81)-42-724 6317.
E-mail address: nemoto@gencom.co.jp (N. Nemoto).

[1] Present address: KAN Research Institute, Science Center Building 3, Shimogyo-ku, Kyoto 600-8815, Japan.

Abbreviations: Cy5-puro, Cy5-conjugated puromycin; Cy5-puro-kinesin, kinesin labeled with Cy5-puro; GFP, green fluorescent protein; AMP-PNP, adenosine 5′-(β,γ-imido) triphosphate

0014-5793/01/$20.00
PII: S0014-5793(01)02581-9

assays and C-terminal labeling of proteins with fluorescent puromycin will be a powerful technique for analyzing the function of proteins encoded by currently uncharacterized genes.

We demonstrate the utility of this technique by analyzing the function of kinesin molecules. Kinesin is a motor protein that transports organelles along microtubule using the energy of the hydrolysis of ATP [13,14]. Conventional kinesin is a dimer of two identical heavy chains, each of which is composed of a head that is responsible for the motor function, and a tail that binds an organelle [15]. Here we synthesized kinesin labeled with Cy5-conjugated puromycin (Cy5-puro-kinesin) at the C-terminus in the cell-free translation system, and visualized its movement along microtubules that were fixed on a glass slide by total internal reflection fluorescence microscopy.

2. Materials and methods

2.1. Synthesis of Cy5-puro

Using the phosphoramidite method, dC-CE-phosphoramidite and 5′-Amino-Modifier 5 (Glen Research) were coupled to puromycin, which was bound to a solid support via the 3′-hydroxyl group and protected with a dimethoxytrityl group at the 5′-hydroxyl and a trifluoroacetyl group at the amino group. After deprotection and purification by reversed-phase HPLC, the resultant puromycin derivative was labeled with Cy5 monofunctional dye (Amersham Pharmacia Biotech). The derivative was labeled almost selectively at the 5′-amino group and Cy5-puro was purified with the reversed-phase HPLC. The structural formula of the Cy5-puro is shown in Fig. 1A.

2.2. Kinesin DNA

The gene encoding the N-terminal 606 amino acids of the *Drosophila* kinesin was used as a PCR template [16]. A kinesin DNA fragment without a stop codon was generated by PCR using primers 5′-TGATA ATACC ATGGC CGCGG AACGA GAGAT TC-3′ (forward) and 5′-CTATG GAGCT CTTGC TGGCA TCCGT GCCAG C-3′ (reverse) to produce *Nco*I and *Sac*I restriction sites, respectively. The PCR product digested with *Nco*I and *Sac*I was inserted into pCITE-2a-c(+) (Novagen), and the construct was verified by sequencing.

2.3. Preparation of Cy5-puro-kinesin

The plasmid linearized with *Sac*I was added to STP3 T7 transcription mix (Novagen) to a final concentration of 10 nM and incubated for 15 min at 30°C. STP3 T7 translation mix (Novagen) and Cy5-puro (final concentration of 30 μM) were then added and incubated for 1 h to synthesize kinesin labeled with Cy5-puro (Cy5-puro-kinesin). Unreacted Cy5-puro was removed by gel filtration with a NAP5 column (Amersham Pharmacia Biotech) equilibrated with a solution containing 30 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 80 mM PIPES (pH 6.8). The sample was separated by SDS–PAGE, and the synthesis of Cy5-puro-kinesin was confirmed by Molecular Imager (Bio-Rad Co.).

2.4. Single-molecule imaging

Tubulin was purified from porcine brain and labeled with tetramethylrhodamine (C-1171, Molecular Probes) or biotin-(AC5)2-Osu (#340-06371, Dojindo) [17]. Tetramethylrhodamine-labeled tubulin was polymerized in the assembly buffer (80 mM PIPES, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 33% (v/v) glycerol, pH 6.8) for 10 min at 37°C, and biotin-labeled tubulin was added and incubated for 15 min more at 37°C. Microtubules thus prepared were stabilized with addition of 4 μM taxol. The labeling ratios of tetramethylrhodamine and biotin to tubulin were 10% and 4%, respectively. For single-molecular imaging, we made a flow cell consisting of a quartz slide and coverslip separated by two slivers of film 50 μm thick. In order to immobilize the microtubules, 15 μM biotinylated bovine serum albumin was infused into the cell. After washing, 170 nM streptavidin and the biotinylated microtubule were successively flowed into the cell to attach the microtubules to the glass surface. The glass surface was then blocked with 1 mg/ml Casein (#073-19, Nakalai) to prevent the non-specific adsorption of kinesin. Cy5-puro-kinesin prepared as described above was diluted 200-fold into a solution containing 80 mM PIPES (pH 6.8), 2 mM ATP, 2 mM $MgCl_2$, 1 mM EGTA, 0.3% (w/v) methylcellulose, and the oxygen scavenger system (25 mM glucose, 2.5 μM glucose oxidase, 10 nM catalase, 10 mM dithiothreitol) [18]. This solution was applied into the flow cell, the slivers of film in flow cell were removed, and then the flow cell was sealed with the nail enamel. Microtubule and Cy5-puro-kinesin was observed by total internal reflection fluorescence microscopy [4,5]. Microtubules were illuminated with a green solid-state laser (2.8 mW, 532 nm, μ-Green Model 4601, Uniphase, USA) and their positions were marked. Cy5-puro-kinesin was illuminated with a He–Ne laser (1.0 mW, 632.8 nm). Images were taken by a SIT camera (C2400-08, Hamamatsu Photonics, Japan) coupled to an image intensifier (VS4-1845, Video Scope International, USA) and recorded on videotapes. Fluorescence images were digitally captured at video rate, and the fluorescence intensity

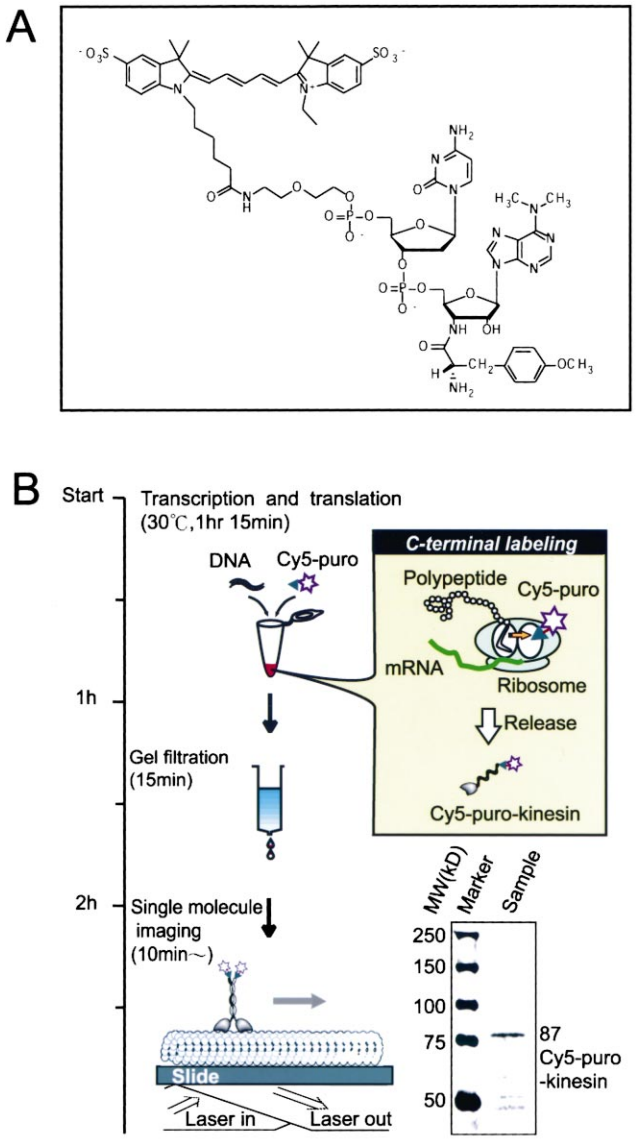

Fig. 1. Synthesis of kinesin labeled with Cy5-puro. A: The structural formula of Cy5-puro. B: A schematic drawing of the procedure for synthesizing and analyzing the function of Cy5-puro-kinesin. The cDNA encoding kinesin without a stop codon was added to reticulocyte lysate, and transcription and translation were performed in vitro. The translated products were analyzed by 7.5% SDS–PAGE and Cy5-puro-kinesin was visualized with a Molecular Imager.

and movement of Cy5-puro-kinesin was analyzed using a program written specifically for this analysis on a Halcon image processor (MVTec Software GmbH, Germany).

3. Results

3.1. Synthesis of Cy5-puro-kinesin

Truncated kinesin molecules (1–606 amino acids) were synthesized from cDNA without a stop codon in the presence of Cy5-puro in the in vitro transcription and translation system (Fig. 1). Cy5-puro-kinesin was obtained after only 2 h of incubation. A single fluorescent band of Cy5-puro-kinesin with the apparent molecular weight of 87 kDa was observed by SDS–PAGE (Fig. 1B). The concentration of Cy5-puro-kinesin was estimated to be 240 nM from the fluorescence intensity of the band.

3.2. Direct observation of individual Cy5-puro-kinesin moving along microtubules

A reticulocyte lysate containing Cy5-puro-kinesin was subjected to gel filtration to remove free Cy5-puro. The kinesin solution containing an oxygen scavenging system was introduced into a flow cell, and observed by total internal reflection fluorescence microscopy at 23°C. Individual Cy5-puro-kinesin molecules could be seen to move along tetramethylrhodamine-labeled microtubules attached to the glass surface (Fig. 2A,B). The average velocity of 0.5 μm/s (Fig. 2C), interaction time of 2.7 ± 0.08 s (data not shown), and run length of 1.2 ± 0.04 μm (Fig. 2D) are all similar to those reported for intact kinesin [5,19]. These results indicate that Cy5-puro labeling had no effect on the motility of kinesin.

3.3. Efficiency of C-terminal labeling with Cy5-puro

Fluorescence intensities of Cy5-puro-kinesin moving along microtubules were quantified to evaluate the labeling ratio of Cy5-puro to kinesin (Fig. 3). The histogram of the fluorescence intensities shows two peaks at 25 and 50 arbitrary units and was well fit by the sum of two Gaussian functions (Fig. 3A). A dimer structure is required for conventional kinesin to move processively along microtubules [5,20]. Thus, the distribution of the fluorescence intensity in Fig. 3A could be attributed to kinesin dimers with one or two Cy5-puro molecules. Next, the photobleaching process of Cy5-puro-kinesin attached to microtubules was observed. When kinesin was mixed with microtubules in the presence of 1 mM AMP-PNP (adenosine 5′-(β,γ-imido) triphosphate), dissociation of kinesin from the microtubules was extremely slow ($k_{off} = 0.001$/s) [21]. We frequently observed two-step photobleaching process; fluorescence intensity decreased from 50 units to 25 units at the first photobleaching of the molecule

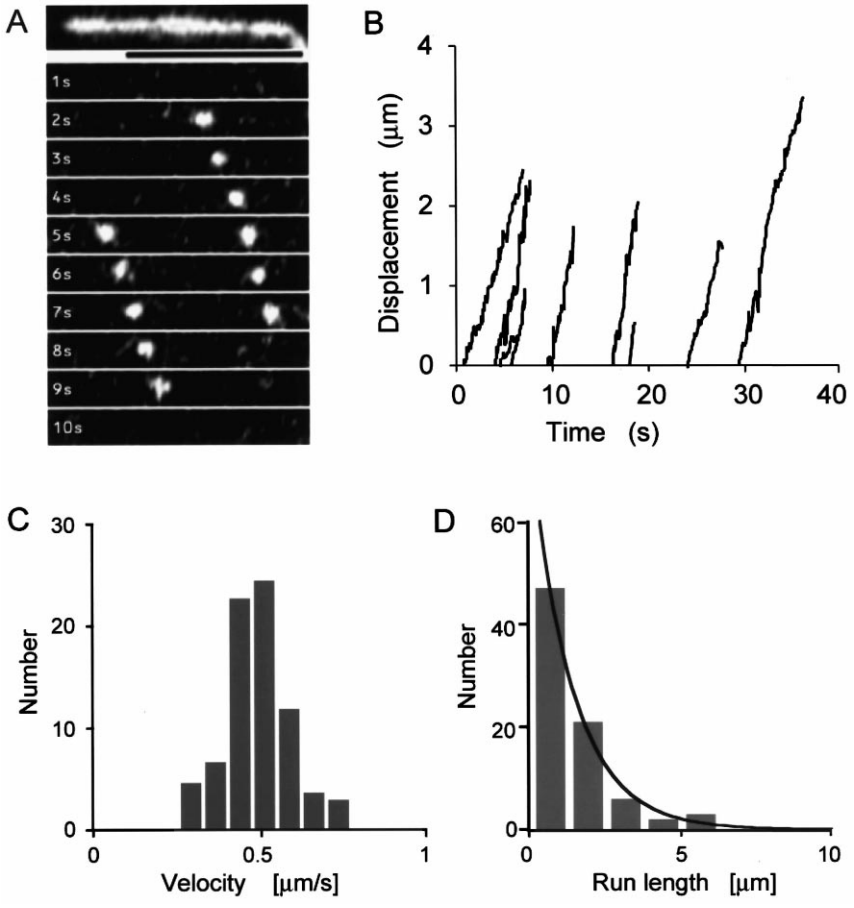

Fig. 2. Movement of single Cy5-puro-kinesin molecules along a microtubule. A: Fluorescence micrographs of a microtubule (top panel) and serial micrographs of Cy5-puro-kinesin molecules (t in s). Scale bar, 5 μm. B: The centers of the fluorescence spots from Cy5-puro-kinesin molecules moving along microtubules ($n = 13$, total length = 130 μm) were analyzed for 40 s, and the displacement from the position of attachment is shown. C: The distribution of the velocity of Cy5-puro-kinesin ($n = 79$). The average velocity was 0.50 μm/s. D: The distribution of the run length of Cy5-puro-kinesin. An exponential curve was fit to the data.

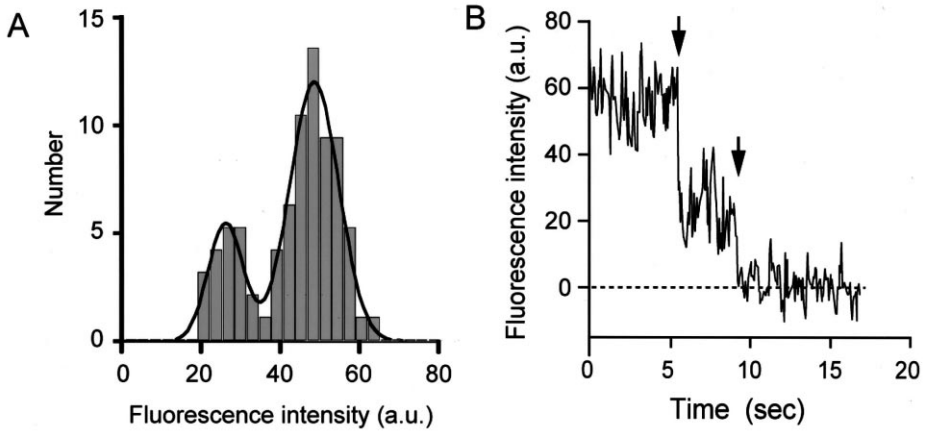

Fig. 3. Fluorescence intensity of Cy5-puro-kinesin. A: The distribution of the fluorescence intensities of Cy5-puro-kinesin moving along microtubules. The solid line indicates the sum of two Gaussian functions fit to the data. B: The time course of the fluorescence intensity of kinesin with two Cy5-puro molecules in the presence of 1 mM AMP-PNP. Photobleaching occurred at the times indicated by the arrows. The broken line indicates the level of background fluorescence.

(the left arrow in Fig. 3B) and then decreased again from 25 to 0 (the right arrow in Fig. 3B). This result confirmed that the peaks on Fig. 3A correspond to the fluorescence signals from single and double Cy5-puro-labeled kinesin dimers, respectively. The populations of each species were calculated from the distribution. The labeling ratio (number of Cy5-puro per single kinesin polypeptide) was determined to be 87%.

4. Discussion

Fluorescence microscopy is a very effective method for studying the interactions of protein molecules, however, attaching chromophores to specific sites on protein molecules without diminishing their function is a major consideration for application of this method. Thus it has been impossible to widely apply microscopy techniques to studies of proteins whose functions are, as yet, unknown.

The method we report here has essential and practical advantages over the methods for fluorescence labeling that are currently available because it allows specific and non-disruptive labeling of any protein of interest. At present, fluorescent labeling of target proteins by fusion to a fluorescent protein such as GFP is widely accepted as the best available approach. The C-terminal labeling of a target protein with puromycin is superior to fusing a target protein to GFP in the following respects. (1) The DNA sequence of the target protein does not have to be modified. Appropriate mRNA lacking a stop codon can be directly prepared from the wild-type gene by PCR. (2) Various kinds of puromycin analogs, such as fluorescent puromycin with various excitation and emission spectra, and biotin–puromycin, are available. These are useful for both single-molecule imaging and for manipulation of samples. (3) As puromycin is small and incorporated only at the C-terminus, it produces little interference to protein function. The molecular weight of puromycin is so smaller than GFP that it significantly reduces the possibility of steric hindrance of protein–protein interactions. (4) Many commonly used variants of GFP are insoluble when expressed, thus proteins of interest are often rendered insoluble upon fusion to GFP [22,23]. The small size of puromycin is unlikely to affect the solubility properties of target proteins.

The preparation time for the functional analysis was only 2 h after the cDNA was added to in vitro transcription and translation system. These results highlight the potential applications of this method to the study of various kinds of protein–protein interactions, which will be required more and more during the post-genomic age.

Acknowledgements: This work was partly supported by the Kowa Life Science Foundation, Novartis Foundation (Japan) for the Promotion of Science. We thank Drs. S. Tsukita, and R. Kamiya for helpful discussion and Dr. J. Hardy for critical reading of the manuscript. We also thank Ms. A. Doi for the preparation of the specimens. Correspondence and requests for materials should be addressed to N.N. (nemoto@gencom.co.jp).

References

[1] International Human Genome Sequencing Consortium, (2001) Nature 409, 860–921.
[2] Venter, J.C. et al. (2001) Science 291, 1304–1351.
[3] Mendelsohn, A.R. and Brent, R. (1999) Science 284, 1948–1950.
[4] Funatsu, T., Harada, Y., Tokunaga, M., Saito, K. and Yanagida, T. (1995) Nature 374, 555–559.
[5] Vale, R.D., Funatsu, T., Pierce, D.W., Romberg, L., Harada, Y. and Yanagida, T. (1996) Nature 380, 451–453.
[6] Weiss, S. (1999) Science 283, 1676–1683.
[7] Mehta, A.D., Rief, M., Spudich, J.A., Smith, D.A. and Simmons, R.M. (1999) Science 283, 1689–1695.
[8] Mason, W.T. (1999) in: Fluorescent and Luminescent Probes: A Practical Guide to Technology for Quantitative Real-time Analysis, Academic Press, New York.
[9] Prasher, D.C. (1995) Trends Genet. 11, 320–323.
[10] Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A. and Tsien, R.Y. (1995) Trends Biochem. Sci. 20, 448–455.
[11] Chalfie, M., Tu, Y., Euskiechen, G., Ward, W.W. and Prasher, D.C. (1994) Science 263, 802–805.
[12] Nemoto, N., Miyamoto-Sato, E. and Yanagawa, H. (1999) FEBS Lett. 462, 43–46.
[13] Vale, R.D. and Milligan, R.A. (2000) Science 288, 88–95.
[14] Hirokawa, N. (1998) Science 279, 519–526.
[15] Vale, R.D. and Goldstein, L.S. (1990) Cell 60, 883–885.
[16] Giniger, E., Wells, W., Jan, L.Y. and Jan, Y.N. (1993) Roux's Arch. Dev. Biol. 202, 112–122.

[17] Hyman, A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L. and Mitchison, T. (1991) Methods Enzymol. 196, 478–485.
[18] Harada, Y., Sakurada, K., Aoki, T., Thomas, D.D. and Yanagida, T. (1990) J. Mol. Biol. 216, 49–68.
[19] Romberg, L., Pierce, D.W. and Vale, R.D. (1998) J. Cell Biol. 140, 1407–1416.
[20] Hancock, W.O. and Howard, J. (1998) J. Cell Biol. 140, 1395–1405.
[21] Hancock, W.O. and Howard, J. (1999) Proc. Natl. Acad. Sci. USA 96, 13147–13152.
[22] Thomas, C.L. and Maule, A.J. (2000) J. Gen. Virol. 81, 1815.
[23] Jakobs, S., Subramaniam, V., Schönle, A., Jovin, T.M. and Hell, S.W. (2000) FEBS Lett. 479, 131–135.