Issue 3, 2006

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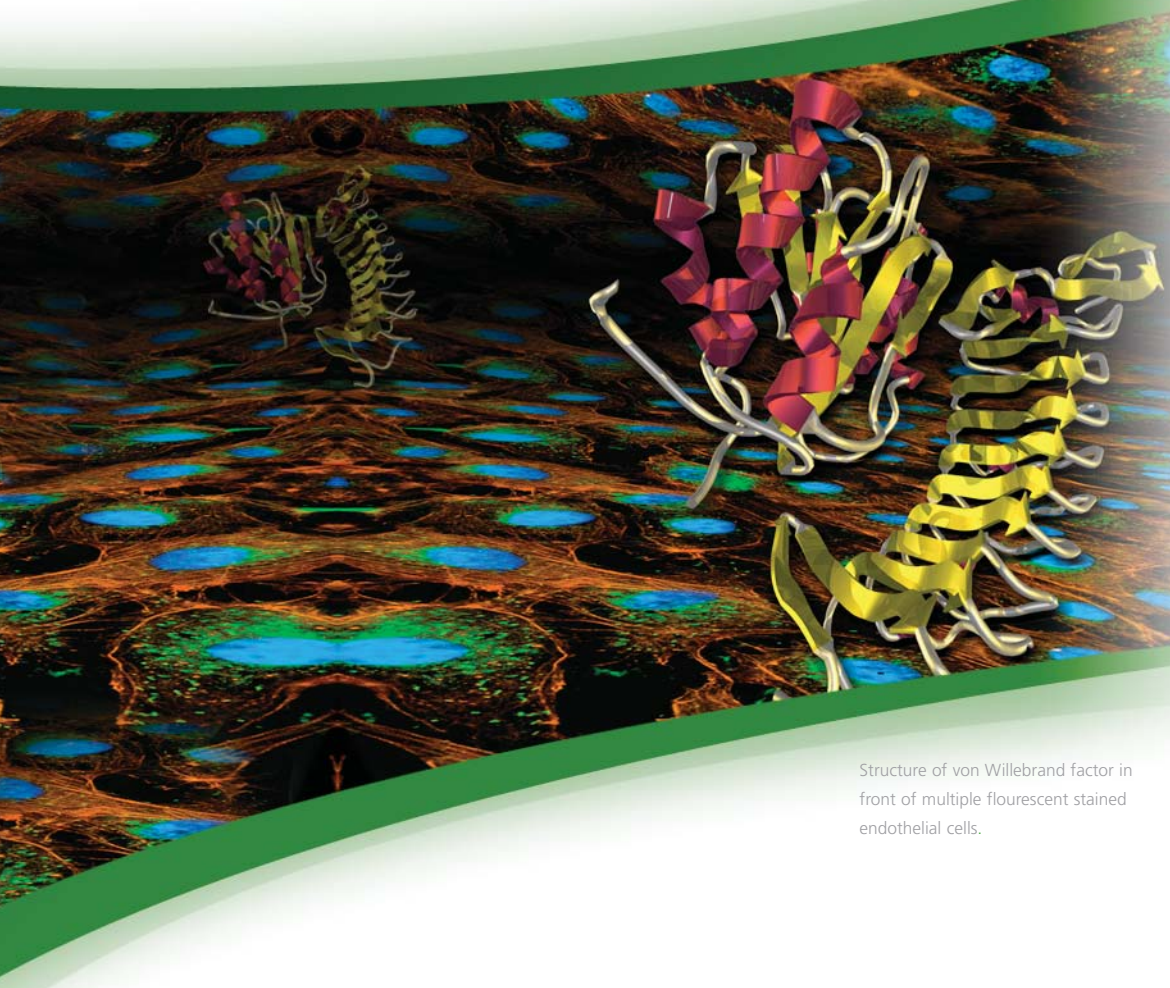

Structure of von Willebrand factor in front of multiple flourescent stained endothelial cells.

NEW FLUORESCENT DYES FOR ELECTROPHORESIS

LABELS AND CONJUGATES

SINGLE MOLECULE DETECTION WITH ATTO 647N NTA

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New Fluorescent Dyes for Electrophoresis

New fluorescent dyes for convenient and reliable detection of proteins on electrophoresis gels

P. Nording, A. Rück, B. Schönenberger (Sigma-Aldrich, Switzerland)

The 1- or 2-dimensional separation of protein mixtures by gel electrophoresis is still one of the most commonly used separation methods. In many cases, qualitative protein identification is of interest. Other analyses may include the quantitative comparison of different protein patterns, such as "differential display" analyses. For further characterization, the bands/spots may be excised and digested for subsequent identification by MS analysis and database search. Protein staining of electrophoresis gels is of great importance to the outcome of such experiments.

Traditional protein stains like Coomassie® Brillant Blue have the advantage of easy handling and good MS compatibility, but often lack the desired sensitivity. Silver stains on the other hand are extremely sensitive, but have the following disadvantages: narrow linear range of quantification, restricted MS compatibility, high time consumption, complicated handling, and laborious waste management. Fluorescent dyes provide advantageous alternatives. They are sensitive, have a broad linear range for quantification, and are MS compatible. Even for the simple staining of 1D-gels, fluorescent dyes offer the advantages of time reduction and ease-of-use.

Lucy dyes for protein staining

The application profiles of 3 recently developed fluorescent dyes for the sensitive detection of proteins on electrophoresis gels, Lucy-506, Lucy-565, and Lucy-569, were analyzed using following parameters:

- sensitivity and limit of detection
- linear dynamic range
- different staining protocols
- optimal electrophoresis conditions
- protocol length and ease-of-handling
- detection and visualization
- compatibility with subsequent MS analysis

All 3 dyes are "stains", e.g., not covalent-binding "labels". The adsorption to the protein is achieved via the SDS-coating, which must not be removed by organic solvents after electrophoresis. Native gels, which were run under SDS-free conditions, may be visualized by incubating them in SDS directly after electrophoresis, prior to staining.

For the application profile analyses, staining was performed according to standard protocols. For the determination of MS compatibility, protein bands were excised from Lucy-stained gels, washed according to common protocols, and digested using trypsin. The resulting peptides were mixed with HCCA matrix, loaded onto a MALDI sample plate, and measured by MS.

Various techniques for detection were investigated, e.g., illuminating the gels on a transilluminator with the corresponding wavelength (Dark-Reader®; UV-Screen) and imaging using a CCD-camera plus filter. Alternatively, a laser scanner may be used, with the respective excitation and emission filter settings, corresponding to the spectral data of the dye used.

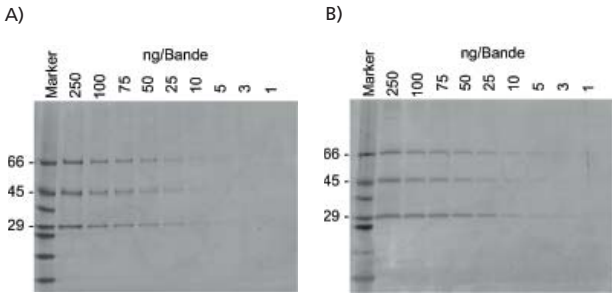

Figure 1. *1D-Mini-gels (BSA, Ovalbumin, Carbonic anhydrase), stained with:*
A) Lucy-506
B) Lucy-565 (Imaging by laser scanner FLA-3000, Fuji).

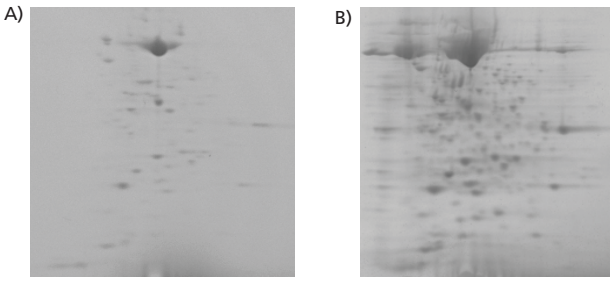

Figure 2. *2D-Mini-gels (10 µg* of E. coli *protein extract), stained with*
A) Coomassie Brillant Blue (Imaging by CCD-Camera)
B) Lucy-506 (Imaging by laser scanner FLA-3000, Fuji).

New Fluorescent Dyes for Electrophoresis

LUCY-506 shows highest sensitivity, with a detection limit of around ~3 ng of protein per band. The standard procedure is a post electrophoretic staining with omission of the fixation step. The stain is completed after 60 minutes and detection is possible immediately thereafter.

LUCY-565 is recommended if, after staining, a Western blot is to be performed from the same gel. This is possible, because staining is done under neutral, non-fixing conditions. With traditional dyes, 2 gels and therefore, double the amount of sample material is necessary.

LUCY-569 is particularly well suited for protein quantitation. It has an extraordinary broad linear dynamic range between 10-6,000 ng/band, which is far larger than most silver staining methods, Coomassie Brillant Blue, or other fluorescent dyes.

These results reflect the standard outcome of experiments, not the very best results selected from large series of experiments.

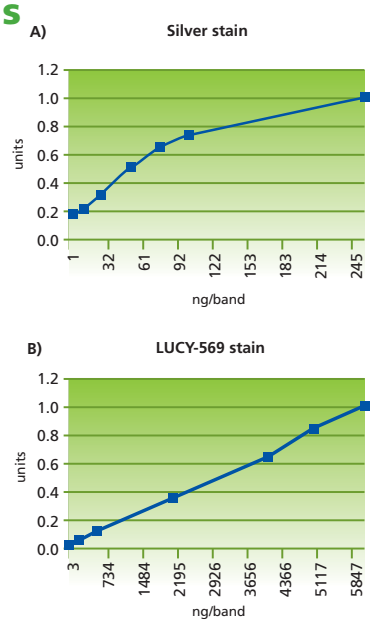

Figure 3. *Linear dynamic range for protein quantitation (Ovalbumin) after:*
A) silver stain
B) Lucy-569-stain.

Stain	Standard Procedure	Prestaining Procedure	Stain After Fixation	Native PAGE Stain	Neutral Stain Before WB
	Post-electrophoretic stain in HOAc; time required: 1 hour	Dye in cathode -running-buffer with subsequent destaining in HOAc; time required: 50-60 minutes.	TCA-fixation; SDS rinse; stain in NaOAc; time required: 1 hour 45 minutes	Run gel SDS-free; rinse gel in SDS; then standard procedure; time required: 1 hour 30 minutes	Postelectrophoretic stain in water; time required: 1 hour; continue with Western blot transfer
Lucy-506	+++	++	+	+++	-
Lucy-565	+	+	++	+++	+++
Lucy-569	+++	++	++	+++	-

Table 1. *Suitability and performance of Lucy stains for various staining procedures.*

Imaging Device	Illumination	Lucy-506 Filter	Lucy-506 Sensitivity	Lucy-565 Filter	Lucy-565 Sensitivity	Lucy-569 Filter	Lucy-569 Sensitivity
Polaroid Camera	Dark Reader® ($\lambda_{max.}$ ~450 nm)	Orange Filter	+		Not tested		Not tested
CCD camera	UV screen ($\lambda_{max.}$ ~310 nm)	590 nm band pass	-	590 nm band pass	++	590 nm band pass	+++
	Dark Reader® ($\lambda_{max.}$ ~450 nm)	590 nm band pass	+++	590 nm band pass	+	590 nm band pass	+
		amber filter	+	amber filter	-	amber filter	-
laser scanner	473 nm laser	520 nm cut off	+++	520 nm cut off	-	520 nm cut off	-
	532 nm laser	580 nm cut off	-	580 nm cut off	+++	580 nm cut off	++

Table 2. *Image devices, methods and performance*

MALDI analysis following SDS electrophoresis

HCCA gold grade matrix was used to investigate the suitability of the new fluorescent protein stains for subsequent MALDI-MS analysis. The 100 ng band of β-galactosidase was excised from a SDS-PAGE gel stained with Lucy-506, washed according to common procedures and digested using a trypsin digestion kit (Cat. No. PP0100). The resulting peptides were placed onto a MALDI target with HCCA and measured with Shimadzu® Kratos CFR instrument (reflectron mode, **Figure 4a**). The same procedure was applied to another gel stained with SYPRO® Ruby. The resulting peaks were used for protein-identification by peptide mass fingerprint. Database-analysis resulted in a sequence coverage of 30% for Lucy-506, and 25% for SYPRO Ruby, using identical search parameters.

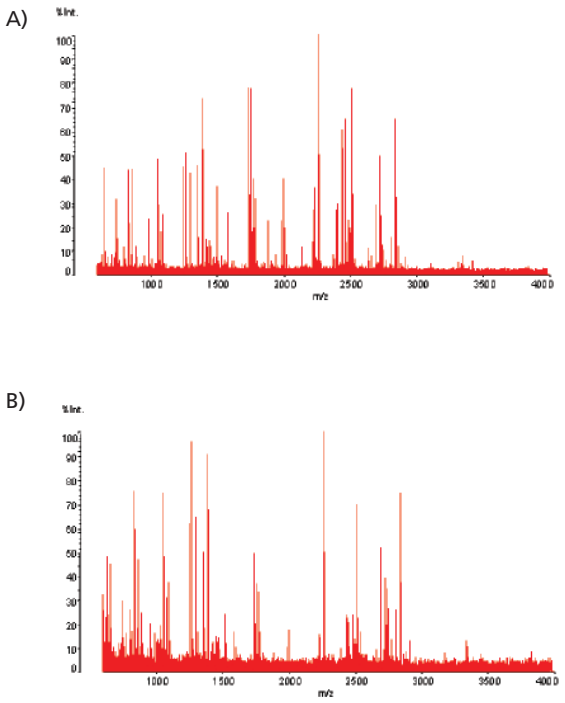

Figure 4. *MALDI peptide mass fingerprint after in-gel-digest: 100 ng of* E. coli *β-galactosidase, separated by SDS-PAGE, stained with (a) Lucy-506 and (b) SYPRO Ruby, after band excision and trypsin-digest. MALDI spectra of extracted peptides, crystallized with HCCA and measured on Shimadzu® Kratos CFR MALDI-instrument in reflectron mode.*

In summary, Lucy-506, Lucy-565, and Lucy-569 are suitable for staining proteins on polyacrylamide gels (both 1D- and 2D-Electrophoresis) and offer reliable, convenient, and economical alternatives to current silver staining techniques or existing fluorescent staining methods for the detection and characterization of minute amounts of protein.

References
White et al., Electrophoresis **25**(17), 3048-3054 (2004)

Ordering Information

Cat. No.	Description	Package Size
14149	Lucy-506 (5000 x stock solution)	500 µl
43772	Lucy-565 (5000 x stock solution)	500 µl
41629	Lucy-569 (5000 x stock solution)	500 µl
PP0100	Trypsin digestion kit	1 kit
70990	α-Cyano-4-hydroxycinnamic acid	250 mg
		1 g
55272	α-Cyano-4-hydroxycinnamic acid	10 x 25 mg
39468	α-Cyano-4-hydroxycinnamic acid	10 x 10 mg

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Labels and Conjugates

Atto labels and their conjugates – versatile, bright and stable tools for imaging

M. Bäumle, P. Nording, B. Schönenberger (Sigma-Aldrich, Switzerland)

Atto dyes are a comprehensive series of fluorescent dyes, covering the entire spectrum of visible light and matching the most common output wavelengths of excitation light sources, especially mercury and xenon lamps, but also common lasers (**Table 2**). Atto dyes provide brightest fluorescence with narrow fluorescence emission spectra. These properties enable the parallel imaging of different targets in cells, tissues, or other biological samples. **Figure 2** shows characteristic absorbance and emission spectra of the Atto dye family.

Atto 488 is a superior alternative to widely used fluorescein, providing tremendously improved photostability and brighter fluorescence. It can be excited with the same light sources as fluorescein dyes or Alexa 488, and the same optical filter sets and instrument settings are used to record the emission. Proteins can be labeled with numerous Atto 488 labels without significant quenching. For longer wavelengths, Atto 550 and Atto 565 are efficiently excited by HeNe lasers (543 nm) and are used as alternatives to rhodamine dyes, Cy™3, or Alexa 550, offering more intense brightness and very good photostability. Atto 635, 647N, or Atto 655 are well suited for excitation with HeNe or Krypton lasers, similar to Cy™5, or Alexa 647.

Autofluorescence of biological samples or support media can be a serious limitation for sensitivity and specificity of fluorescent techniques like immunohisto chemistry and ELISA assays. As most of the fluorescence of biological samples occurs at shorter wavelength, the impact of autofluorescence decreases with longer excitation and detection wavelengths. Similar holds for most of the autofluorescence of solvents, glass, or polymer supports. With excitation maxima up to 740 nm and emission maxima up to 764 nm, Atto dyes provide a set of tools to circumvent problems with autofluorescence.

Atto labels are available as reactive succinimidyl esters and maleimides, enabling straight-forward coupling by common procedures. For additional information on individual products please visit our Web site at:
sigma-aldrich.com/fluorescent_labels.

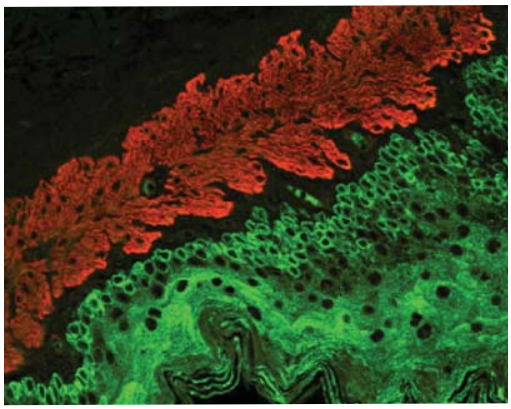


Figure 1. *Rat stomach: Actin stained with mouse anti–smooth muscle α-actin antibody and Atto 488 anti-mouse IgG (green), cytokeratin stained with polyclonal rabbit anti-cytokeratin and Mega 485 anti-rabbit IgG (yellow), both labels are excited by just one laser (Argon laser).*

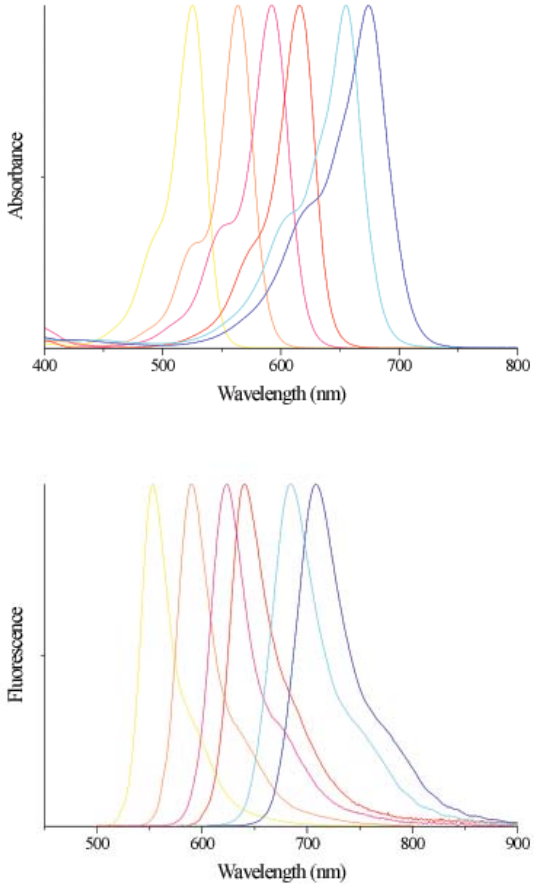


Figure 2. *Absorption and emission spectra of Atto 520, Atto 565, Atto 590, Atto 610, Atto 655 and Atto 680*

Antibody Conjugates Selection Guide

Label	Goat anti-mouse IgG	Goat anti-rabbit IgG	Goat anti-human IgG	Rabbit anti-chicken IgG
Atto 488	18772	**62197**	52526	
Atto 550	43394	*__44328__		
Atto 590		**68919**		50913
Atto 647N	50185	**40839**		
Mega 485	12708	*__38376__		
Mega 520	39304	*__02295__		

Table 1: *Antibody conjugates labeled with new Atto- or Mega-labels*

For order information see page 7

* Available Soon

Name	λ_{max} abs [nm]	λ_{max} em [nm]	ηfl [%]	λ_{max} [l/mol cm]	Suitable Laser Excitation Sources	Spectral Match
Atto 465	449	503	55	75000	Ar laser	
Atto 488	501	523	80	90000	Ar laser	FITC, Alexa 488
Atto 495	499	535	45	80000	Ar laser	
Atto 520	525	545	90	110000	Ar laser	JOE
Atto 532	532	553	90	115000	Ar laser	Alexa 532
Atto 550	554	576	80	120000	HeNe laser	TRITC, Cy™3, Alexa 546
Atto 565	563	592	92	120000	HeNe laser	Rhodamine B, Lissamine, Alexa 555
Atto 590	598	634	80	120000	HeNe laser	Texas Red, Alexa 594
Atto 594	601	627	85	120000	HeNe laser	Texas Red, Alexa 594
Atto 610	605	646	70	150000	HeNe laser	Alexa 610
Atto 620	620	641	50	120000	HeNe laser	
Atto 633	629	657	64	130000	HeNe laser, Ruby laser	Alexa 635
Atto 635	635	659	25	120000	HeNe laser, Ruby laser	Alexa 635
Atto 647N	645	673	20	120000	HeNe laser, Kr laser, Ruby laser,	Cy™5, Alexa 647
Atto 655	665	690	30	125000	HeNe laser, Kr laser Ruby laser, GaInP laser	
Atto 680	680	702	30	125000	HeNe laser, Kr laser, GaInP laser	Cy™5.5, Alexa 680
Atto 700	740	764	10	120000	Ruby laser	Alexa 700

Table 2: *Atto labels and their spectroscopic properties*

Antibody and other fluorescent conjugates:

We optimized the labeling of antibodies based on the innovative series of Atto labels, to provide high quality conjugates with ideal brightness and low background.

Secondary antibodies, the general work horses for immuno-chemistry, are offered as conjugates with several of our Atto labels as well as Mega labels, which are characterized by a large gap between excitation and emission maxima. (**Tables 1 and 3**). For most of the Atto labels, streptavidin and biotin conjugates are also available.

Multiple staining with single excitation light:

Parallel staining of different structures or target molecules in biological tissues or other samples can be complicated by overlaps of fluorescence signals. Thus labels with clearly distinct emission spectra are prefered. For this purpose the wide spectrum of labels offers a variety of suitable combinations (**Figure 1-4**)

Where laser light is used for excitation, as in the case of confocal microscopy, the suitable excitation of labels with distinct emission spectra may require two or more different lasers, because for most common labels (e.g., FITC, TRITC, (Cy™3, Cy™5, Alexa labels) excitation and emission maxima are relatively close. For efficient fluorescence imaging, the laser wavelength must be close to the excitation maximum wavelength of these dyes. However, each additional laser source increases costs significantly, thus multiple fluorescent staining with just one excitation source would be a convenient and cost efficient methodology and provide increased flexibility.

Another potential problem with multiple fluorescent staining is the overlap of emission wavelength of dye (A) with excitation of dye (B), resulting in a lower signal intensity of dye A.

In contrast to above mentioned labels Mega labels are characterized by a large gap between excitation and emission wavelength (**Table 3**). All of them can be excited with argon lasers or other widely used light sources, but their fluorescence varies. Mega labels, if combined with Atto dyes, enable the visualization of different structures with just one excitation source. **Figure 1** shows an application of an Atto 488 and a Mega 485 labeled antibody in confocal microscopy. But such combinations can also be used on conventional fluorescence microscopes, using mercury lamp, even using standard filter sets optimized for conventional fluorophores (**Figure 3**).

Name	λ_{max} abs [nm]	λ_{max} em [nm]	λ_{max} [l/mol cm]
Fluorescent Red Mega 480	480	640	40000
Fluorescent Red Mega 485	485	559	20000
Fluorescent Red Mega 500	500	612	90000
Fluorescent Red Mega 520	520	664	50000

Table 3: *Mega labels and their spectroscopic properties*

Labels and Conjugates

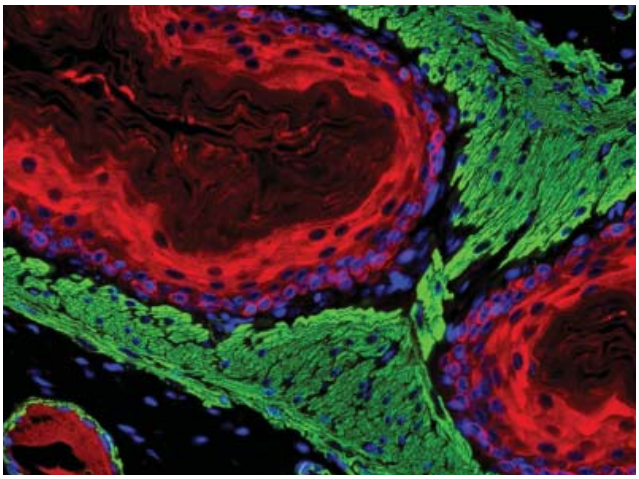
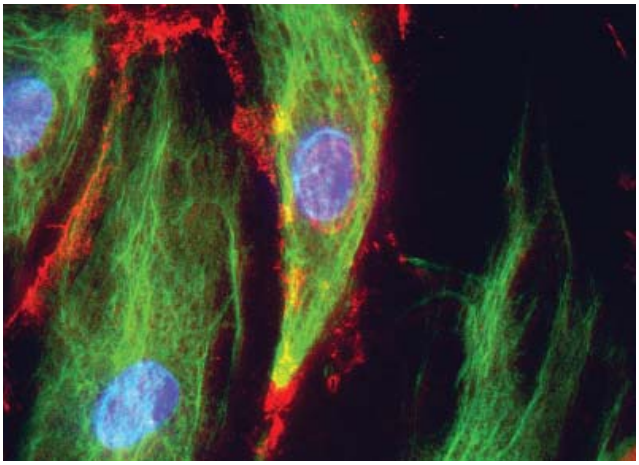


Figure 3: *Rat stomach: Actin stained with mouse anti–smooth muscle α-actin antibody and Atto 488 anti-mouse IgG (green), cytokeratin stained with polyclonal rabbit anti-cytokeratin and Mega 520 anti-rabbit IgG (red), counterstained with DAPI (blue). Courtesy of Jacob Zbaeren, Inselspital Bern, Switzerland.*



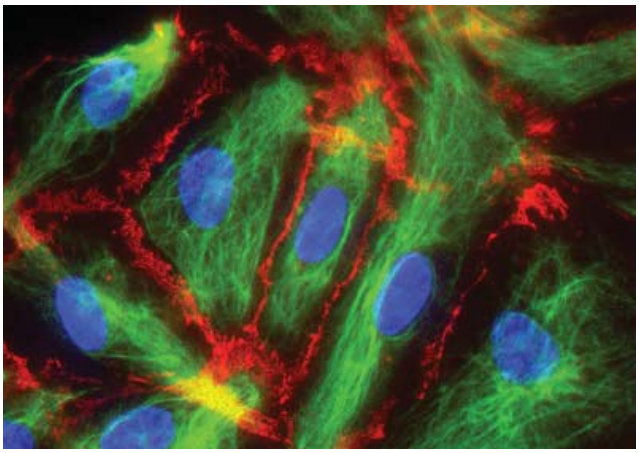


Figure 4. *Human endothelial cells: Vimentin stained with mouse anti-vimentin and Atto 550 anti-mouse IgG (green), cadherine stained with rabbit anti-cadherine antibody and Atto 655 goat anti-rabbit IgG (red). Counterstained with DAPI (blue).*

Stability

For well-known and commonly used labels like fluoresceins, photostability is limited. In various applications, especially in immunofluorescence, bleaching of fluorescence intensity is a major concern, limiting quality and sensitivity of imaging. Photostability becomes even more important with the increasing use of laser excitation, confocal and two-photon illumination, and the increasing sensitivity of methods down to the single molecule level. Also the tracking of processes over time in living cells requires stable dyes.

Atto labels, in contrast to some of the most widely used dyes, have more rigid structures, which makes them more photostable. Atto conjugates are exceptionally stable, in several cases even outperforming the dyes, that were considered the best choice until now.

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Separating Structural Heterogeneities from Stochastic Variations in Fluorescence Resonance Energy Transfer Distributions via Photon Distribution Analysis: M. Antonik, et. al., J. Phys. Chem. B., **110**, 6970-6978 (2006)

Ordering Information

Cat. No.	Description	Package Size
18772	Anti-Rabbit IgG - Atto 488 from goat antiserum	1 ml
43394	Anti-Mouse IgG - Atto 550 from goat antiserum	1 ml
50185	Anti-Mouse IgG - Atto 647N from goat antiserum	1 ml
12708	Anti-Mouse IgG - Mega 485 from goat antiserum	1 ml
39304	Anti-Mouse IgG - Mega 520 from goat antiserum	1 ml
62197	Anti-Mouse IgG - Atto 488 from goat antiserum	1 ml
78102	Anti-Mouse IgG - Atto 633 from goat antiserum	1 ml
36098	Anti-Rabbit IgG F (ab')2 fragment - Atto 488, from goat antiserum	1 ml
43328	Anti-Rabbit IgG - Atto 550 from goat antiserum	1 ml
68919	Anti-Rabbit IgG - Atto 590 from goat antiserum	1 ml
40839	Anti-Rabbit IgG - Atto 647N from goat antiserum	1 ml
38376	Anti-Rabbit IgG - Mega 520 from goat antiserum	1 ml
02295	Anti-Rabbit IgG - Mega 485 from goat antiserum	1 ml
52526	Anti-Human IgG - Atto 488 from goat antiserum	1 ml
50913	Anti-Chicken IgG - Atto 590 from rabbit antiserum	1 ml
28616	Atto 425-Biotin	1 mg
49349	Atto 425-maleimide	1 mg
16805	Atto 425-NHS ester	1 mg
09260	Atto 425-Streptavidin	1 mg
50712	Atto 465	1 mg
55607	Atto 465-maleimide	1 mg
53404	Atto 465-NHS ester	1 mg
41051	Atto 488	1 mg
41698	Atto 488-NHS ester	1 mg
16951	Atto 495	1 mg
41022	Atto 495-maleimide	1 mg
00379	Atto 495-NHS ester	1 mg
70706	Atto 520	1 mg
01632	Atto 520-Biotin	1 mg
16590	Atto 520-maleimide	1 mg
77810	Atto 520-NHS ester	1 mg
88032	Atto 525-NHS ester	1 mg
06699	Atto 532	1 mg
88793	Atto 532-NHS ester	1 mg
40592	Atto 540 Q	1 mg
62453	Atto 540 Q-maleimide	1 mg
61683	Atto 540 Q-NHS ester	1 mg
42424	Atto 550	1 mg
92835	Atto 550-NHS ester	1 mg
75784	Atto 565	1 mg
92637	Atto 565-Biotin	1 mg
18507	Atto 565-maleimide	1 mg
72464	Atto 565-NHS ester	1 mg
56304	Atto 565-Streptavidin	1 mg
03722	Atto 580 Q	1 mg
68152	Atto 580 Q-maleimide	1 mg
44756	Atto 580 Q-NHS ester	1 mg
70425	Atto 590	1 mg
43208	Atto 590-Biotin	1 mg
39887	Atto 590-maleimide	1 mg
79636	Atto 590-NHS ester	1 mg
40709	Atto 590-Streptavidin	1 mg
78493	Atto 610	1 mg
43292	Atto 610-Biotin	1 mg
93259	Atto 610-NHS ester	1 mg
41061	Atto 610-maleimide	1 mg
56767	Atto 610-Streptavidin	1 mg
04327	Atto 612 Q	1 mg
50332	Atto 612 Q-maleimide	1 mg
53988	Atto 612 Q-NHS ester	1 mg
92716	Atto 620	1 mg
67351	Atto 620-NHS ester	1 mg
01464	Atto 633-NHS ester	1 mg
08968	Atto 635	1 mg
97875	Atto 647	1 mg
07376	Atto 647-NHS ester	1 mg
07376	Atto 647N-NHS ester	1 mg
41784	Atto 647N-maleimide	1 mg
30700	Atto 647N-Biotin	1 mg
04507	Atto 647N	1 mg
18373	Atto 647N-NHS ester	1 mg
05316	Atto 647N-maleimide	1 mg
93711	Atto 655	1 mg
06966	Atto 655-Biotin	1 mg
80661	Atto 655-maleimide	1 mg
76245	Atto 655-NHS ester	1 mg
02744	Atto 655-Streptavidin	1 mg
94875	Atto 680	1 mg
04971	Atto 680-maleimide	1 mg
16630	Atto 680-Streptavidin	1 mg
30674	Atto 700	1 mg
50611	Atto 700-maleimide	1 mg
16986	Atto 700-NHS ester	1 mg
41087	Fluorescent Red Mega 480	1 mg
55536	Fluorescent Red Mega 480-NHS ester	1 mg
30487	Fluorescent Red Mega 485	1 mg
68360	Fluorescent Red Mega 485-NHS ester	1 mg
13194	Fluorescent Red Mega 490	1 mg
30543	Fluorescent Red Mega 490-NHS ester	1 mg
56900	Fluorescent Red Mega 500	1 mg
08087	Fluorescent Red Mega 500-NHS ester	1 mg
41306	Fluorescent Red Mega 520	1 mg
68493	Fluorescent Red Mega 520-NHS ester	1 mg

Single Molecule Detection with Atto 647N NTA

Significant improvement of single molecule tracking by using superior fluorescent NTA-Atto 647N conjugate

Ruud Hovius, Emmanuel Guignet, Jean-Manuel Segura, Joachim Piguet & Horst Vogel (Institute de Science Biomoleculaire, EPFL, Switzerland), Monika Bäumle (Sigma-Aldrich Switzerland)

- Atto 647N is a superior fluorescent dye for single molecule tracking experiments
- Excellent photostability of the label enables the acquisition of a significant increase in information content

General advantages of fluorescent NTA conjugates

Fluorescent NTA conjugates have been used to detect oligohistidine tagged proteins on blots, in gels, or in solution (**Figure 1**). Recently, this approach has been extended to the detection and characterization of proteins in living cells (Guignet 2004).

Major advantages of this labeling method are:

- the flexibility to use the fluorophore best suited for the application - the nature of the fluorophore has little affect on the binding characteristics of the NTA-probe to the His-tag. The dissociation constants observed for the binding to hexa and decahistidine tagged green fluorescent protein in solution are in the range of 5 to 0.5 µM.
- very fast kinetics - labeling in cells was demonstrated to be complete within 30 seconds and fully reversible within a few minutes upon addition of a strong chelator.
- the reversibility of the interaction allows recovery of the sample.

Single molecule imaging – Atto 647N has excellent photostability

The detection of oligohistidine tagged proteins often involves large populations of target molecules; however, another important application of the NTA-labeling method is the imaging of a single fluorescently labeled molecule in live cells. In single molecule imaging the fluorescent labels are exposed to strong irradiation. Under these conditions most common labels undergo relatively rapid photodestruction, limiting the number of images that can be gathered. Usually, 10-25 images can be acquired with wide-field illumination before bleaching occurs, resulting in short traces of the target molecule's displacement. This in turn has a strong impact on the information that can be extracted. For instance, the mean square displacement analysis of a molecule of which ***n*** images have been recorded will contain [1+ ...+ (***n***-1)] data points. For example, one trace composed of 30 images will give ten times more data points than a trace of 10 images.

Atto 647N has great potential for single molecule studies due to its great photostability and brightness. In a comparative experiment, the ionotropic serotonin receptor expressed in mammalian cells was labeled with either a Cy™5 or an Atto 647N probe. Single molecule imaging revealed the Atto 647N was more photostable than Cy5, having a 2-fold lower bleaching rate (**Figure 2**). The Atto 647N routinely allowed acquisition of films of 50 to 100 frames.

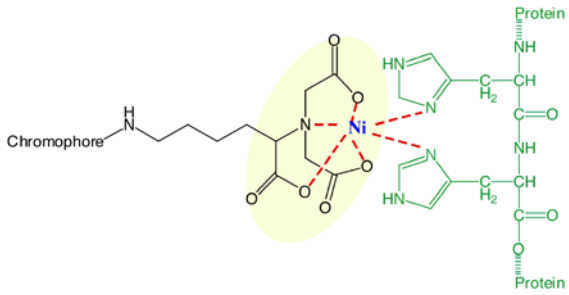


Figure 1. *Interaction of a Ni-NTA-chromophore with an oligohistidine tagged protein.*

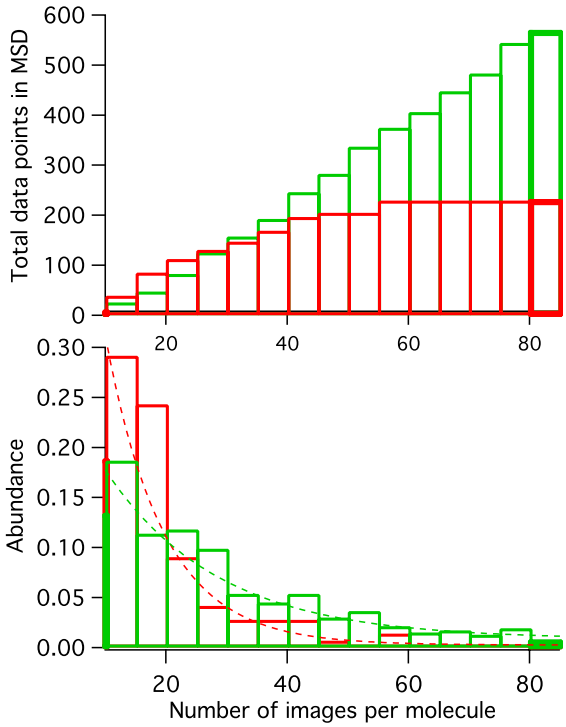


Figure 2. *Atto 647N (green) is more photostable than Cy5 (red).*

Top panel: The accumulative number of data points per molecule observed with Cy5 labeled molecules levels at approximately 200 for a maximal length of 55 frames. However Atto 647N delivers 3-fold more data points due to trace lengths up to 85 images.

Bottom panel: Trace length histogram for a Cy5 (red) and Atto 647N (green) labeled serotonin receptor probe shows that Cy5 undergoes photobleaching twice as fast as Atto 647N.

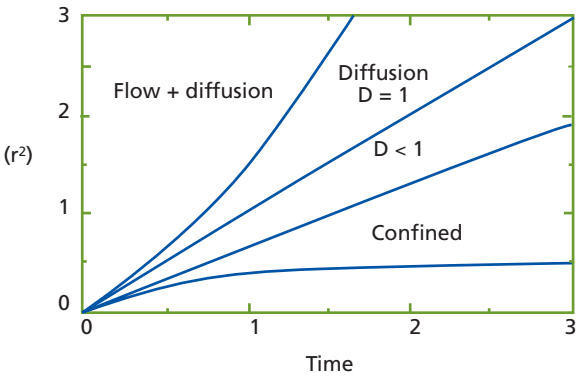
Atto 647N yields very long traces of diffusing single molecules

Films with long traces allow accurate description of the diffusional behaviour of an individual labeled molecule. Routinely for films with short traces, diffusional behaviour is evaluated by plotting the mean square displacement (MSD) observed in a series of relatively short films, each in the range of 12 frames, for a population of molecules versus time (**Figure 3A**). Depending on the type of diffusion a different dependence of the MSD on time is found.

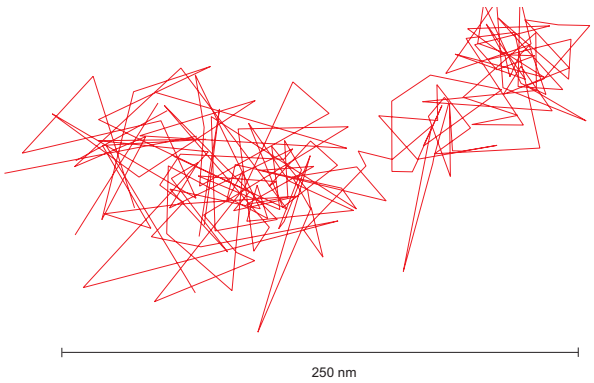
The excellent properties of Atto 647N enable acquisition of image sequences up to approximately 100 frames (an example of 104 frames is shown in **Figure 3B**). Traces of this length allow the evaluation of the MSD of an individual particle, revealing details of its diffusional behaviour. If acquisition had been limited to 12 frames only, then one would have concluded that the diffusion of the particle was within a restricted area (confinement). Evaluation of the longer trace clearly indicates free Brownian diffusion (**Figure 3C**).

These observations are greatly facilitated by the application of very photostable and bright fluorophores like Atto 647N.

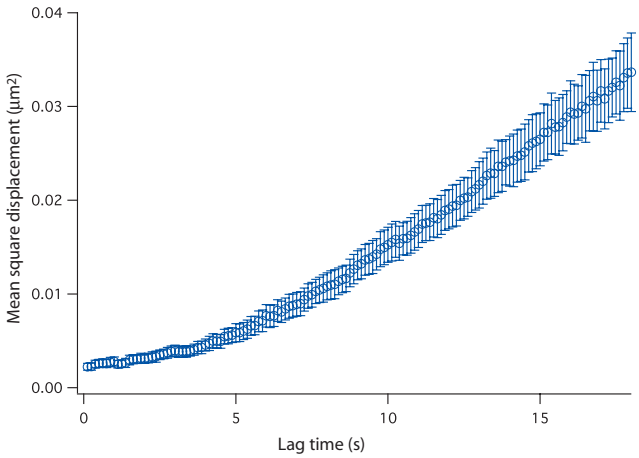
Evaluation of the diffusion of single molecule tracking



3A. *Mean square displacement (MSD) of target molecules having different diffusion modes. Freely diffusing molecules feature an MSD (D=1) proportional with time: MSD ∝ $t^{n=1}$. Molecules whose diffusion is hindered by obstacles (D<1) or restricted to an area (confined) feature an MSD ∝ $t^{n<1}$. Molecules going with a flow or being directionally transported show an MSD ∝ $t^{n>1}$.*



3B. *Single molecule trace of an NTA-Atto 647 labeled serotonin receptor in the plasma membrane of a HEK293 cell (scale bar is 250 nm). 104 frames were recorded for this single molecule.*



3C. *Mean square displacement (MSD) of the trace shown in Figure 3B., indicating that this molecule undergoes free diffusional behavior.*

Ordering Information

Cat. No.	Description	Package Size
04507	Atto 647N	1 mg
18373	Atto 647N-NHS ester	1 mg
05316	Atto 647N-maleimide	1 mg
39625	*NTA-Atto 488	250 µg
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02175	*NTA-Atto 647N	250 µg

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