

Atto Labels – A Challenging Range of New Fluorescent Labels

Fluorescent detection methods are resulting in major improvements in bioanalytical applications because of their extraordinary sensitivity and selectivity. Many methods, especially, radioactive assays, are being replaced, also eliminating their potential health risks and waste disposal problems. Since many target molecules do not possess appropriate fluorescent properties of their own, staining or labeling (covalently) with special fluorophores has become crucial for many different technologies, e.g., microscopy, flow cytometry, electrophoresis and chromatography.



tion, the photostability of fluorescent dyes is crucial for the statistical accuracy of single-molecule detection and for the image quality of scanning confocal microscopy [1]. Fluka is pleased to introduce a series of fluorophores for labeling of biomolecules developed by Prof. K.H.

Drexhage which are designed for the most sensitive applications. A unique combination of properties makes them highly favorable tools for the majority of labeling applications. And some of these properties make them especially suitable for single-molecule detection (figure 2, figure 3). They exhibit very little triplet formation and the long wavelength labels have proven to be more photostable than other commonly used dyes.

Single-Molecule Detection

One of the most exciting aspects of fluorescence technologies is their ability to support decreasing sample sizes down to the single-molecule detection level, which in turn provides the opportunity for miniaturization and high throughput screening. This improvement is based on sophisticated instrumentation and extended data processing capabilities, but is also dependent on appropriate labels. Most established fluorophores suffer from one or more limitations which restrict their functionality in this field. One of the most serious limitations is the formation of triplets. Sensitive single-molecule detection (SMD) requires the formation of high numbers of photons within short time periods. If a part of the excited molecule forms the stable non-emitting triplet state, the yield of photons decreases dramatically. In addition,

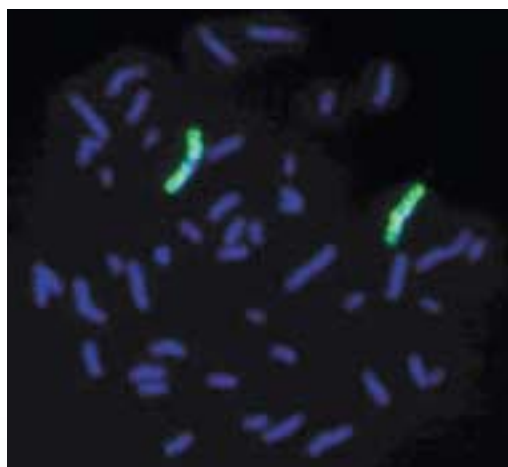


Figure 1: FISH micrograph of biotinylated probes hybridized to chromosome 1 and detected by Fluorescent red 630-avidin conjugate (Cy5-channel), [4].

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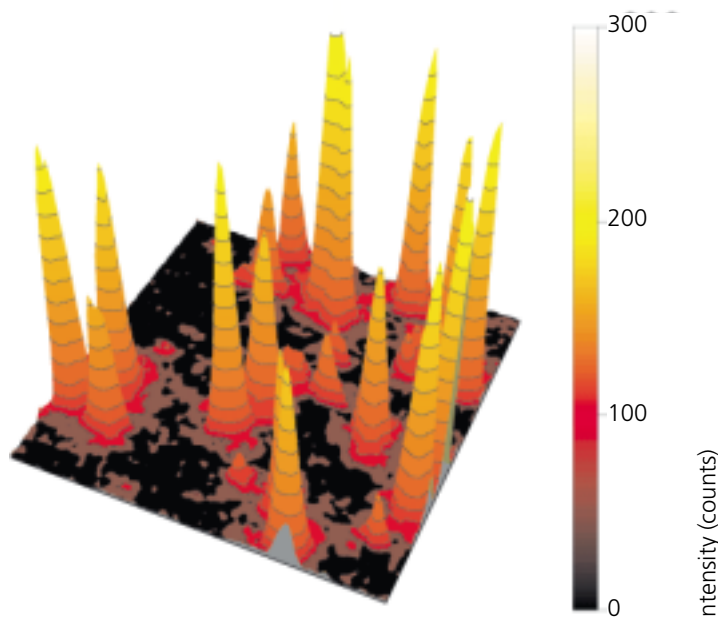


Figure 2: Fluorescence intensity profile of single dye molecules (Atto 650) on a glass surface. The image was acquired on a confocal sample scanning microscope (x100 oil immersion objective, 50 μm pinhole, 10 x 10 μm^2 , 50 nm/pixel) utilizing a pulsed diode laser system (80 MHz, 635 nm, $\sim 5 \mu\text{W}$) in combination with a time correlated single photon counting system (TCSPC).

Our new range of labels show excitation and emission maxima spanning the range from 520 – 680 nm (table 1, figure 4). This provides the opportunity to use a wide variety of excitation sources (table 2) and to match the wavelengths of many commonly used filter sets (table 3). One can also utilize simultaneous multicolor detection. All of our new labels are characterized by high molar extinction coefficients (around 120 000 $\text{M}^{-1} \text{cm}^{-1}$) and high quantum yields (table 1), resulting in a brighter fluorescence. The Stokes' Shift is between 20 and 30 nm. Such shifts are important in avoiding the self-quenching effects observed with other labels, e.g. Bodipy labels [2]. Atto labels are based on rigid structures and do not show any cis-trans isomerisation, as is typically observed with cyanin-based fluorophores. In the case of cis-trans isomerisation of fluorescent labels, only the trans isomers are fluorescent. The cis-isomer does not contribute to the fluorescent signal [3]. Unfortunately, if such a label is excited, the balance may even shift towards the cis-isomer. This can cause problems in applications like fluorescent resonant energy transfer (FRET) or single-molecule detection. Cis-trans equilibrium is

also dependent on environment which can lead to changes of spectroscopic properties through conjugation. The absence of cis-trans isomerisation circumvents these problems encountered with commonly used dyes. The small size of our labels (MW between 400 and 600) minimizes the risk of unwanted steric effects in the interaction of labeled molecules with target structures. Furthermore Atto labels are quite insensitive against pH fluctuation – most of them do not show any effects between pH 2 and 10, and feature low unspecific absorption. The combination of these properties make them excellent tools for all labeling applications.

Atto labels are provided both as free carboxylic acids (used for carboxy coupling) and succinimidyl (NHS) esters. These can be used following the most common procedures for coupling to amino groups. Data sheets including labeling protocols are available at www.sigma-aldrich.com/analytix.

Another general advantage of these dyes is their stability. Chemical stability is better than for many cyanin-based dyes, so you can count on the high purity and activity of these labels.

Conjugates and customized products

All these Atto labels can be also supplied as maleimides, avidin, streptavidin or biotin conjugates. We are ready to ship these conjugates down to the size of 1 mg. A continuous flow of new Atto labels will help you to exploit the full potential of fluorescence based techniques. Moreover we have extensive experience in the synthesis of fluorophores and we are ready to provide custom manufactured or packaged products. If you should have a specific need for fluorophores which is not matched optimally by our catalog products, please contact Pierre Nording at pnording@sial.com. For local pricing please check our homepage or send us an e-mail.

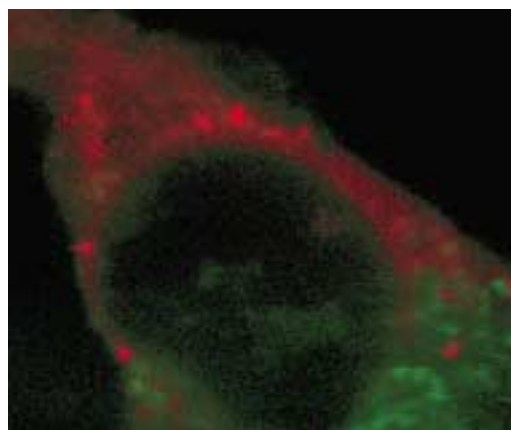


Figure 3: Pseudo color image of the normalized fluorescence intensities of a 3T3-Fibroblast stained with two Atto dyes differing in their fluorescence lifetimes (red: ~ 1.9 ns, green: ~ 3.0 ns, in water). The image was acquired on a confocal sample scanning microscope. The sample was excited with a pulsed diode laser system (80 MHz) at 635 nm, the fluorescence lifetime information was collected above 640 nm by time correlated single photon counting (TCSPC) with a single avalanche photo diode (APD).

New Hemicyanine Dyes optimized for Excitation at 633 nm

New hemicyanine dyes Fluorescent red 630 and Fluorescent red 635 developed by Dyomics match excitation by He-Ne laser line at 633 nm, Argon or Argon-Krypton laser lines at 647 nm and diode lasers (635-650 nm). The structural difference between the two dyes is the bridging of the aminoalkyl group to the neighboring phenyl ring in the benzopyran moiety of Fluorescent red 635. This structure variation causes a slight red shift in absorption. As NHS esters they are generally suitable as nucleic acid or protein labels. Both exhibit fairly low quantum yields if dissolved in aqueous media, but after coupling to a target molecule signal intensity can rise up to one order of magnitude as demonstrated for protein conjugates and labeled oligomers (table 4).

Figure 1 gives an example for a fluorescent in situ hybridization (FISH) application. For FISH no amplification was needed and fluorescence intensity proved to be stable for weeks. These dyes feature also a superior solid state fluorescence. This makes them certainly useful to label DNA for preparation of glass arrays. We will soon add more products based on related chemical structures to cover the other commonly used excitation sources.

... and more new labels ...

For excellent sensitivity you may also want to look at activated, biotinylated or streptavidin-linked phycoerythrin, phycocyanin, and CryptoFluor™ dyes, (™Martek Biosciences Corporation, Columbia, MD, USA). The phycobiliproteins phycoerythrin and phycocyanin, large complexes extracted from algae, are the first choice for applications that require either high sensitivity or simultaneous multicolor detection, e.g. flow cytometry and immunoassay. They provide quantum yields near 100 % and extinction coefficients up to 2.4 million M⁻¹cm⁻¹. The fluorescence yield of a single molecule is equivalent to at least 30 unquenched fluorescein or 100 rhodamine molecules at comparable wavelengths. CryptoFluor™ dyes are phycobiliproteins isolated from cryp-

Table 1: Properties of Atto dyes measured in water (ethanol)

Product	ϵ [M ⁻¹ cm ⁻¹]	λ_{\max} abs [nm]	λ_{\max} em [nm]	η_{fl} [%]	τ_{fl} [ns]
Atto 520	110 000	520 (525)	542 (547)	90 (95)	3.6 (3.7)
Atto 565	120 000	561 (566)	585 (590)	92 (97)	3.4 (3.6)
Atto 590	120 000	598 (598)	634 (634)	80 (90)	3.7 (4.0)
Atto 610	110 000	605 (616)	646 (630)	70 (70)	3.3 (3.3)
Atto 655	110 000	665 (655)	690 (680)	30 (50)	1.9 (3.6)
Atto 680	120 000	680 (675)	702 (699)	30 (40)	1.8 (3.4)

Table 2: Common light sources and suitable Atto dyes

Light source	Main lines [nm]	Best suited Atto labels
Mercury Arc Lamps	365, 405, 436, 546, 577	Atto 565
Xenon Arc Lamps	Continuum and peaks >800 nm	Atto 610, Atto 655, Atto 680
Halogen Lamps	Little UV and violet emission; higher intensity towards longer wavelengths	Atto 610, Atto 655, Atto 680
Argon Ion Laser	488, 514	Atto 520
Argon Krypton Laser	488, 514, 647, 676	Atto 520, Atto 655, Atto 680
Krypton Laser	647, 676	Atto 655, Atto 680
He-Ne Laser	544, 594, 633	Atto 655

Table 3: Common fluorescent labels and Atto dyes matching same emission light filter set-up

Common labels	Matching Atto labels
JOE, Carboxyrhodamine 6G, Alexa 532	Atto 520
TRITC, ROX, Phycoerythrin, Lissamine, Cy 3, Alexa 546	Atto 565
Texas Red, TAMRA, Alexa 594	Atto 590
Phycocyanin	Atto 610
Cy 5, Alexa 674	Atto 655
Cy 5.5, Alexa 680	Atto 680

tomonad algae that provide additional spectral characteristics (λ_{\max} abs 585 nm, λ_{\max} fl 658 nm for CryptoFluor™ Crimson and λ_{\max} abs 566 nm, λ_{\max} fl 600 nm for CryptoFluor™ Gold) and less bulky sizes (MW 40 200, 30 800).

We are also extending our range of widely used carboxyfluorescein (FAM), carboxy-X-rhodamine (ROX) and carboxy-tertamethylrhodamin (TAMRA) derivatives by single-isomer variants of both the carboxylic acids and their NHS-esters. Please find an overview on page 5 of this issue.

	FR 630	FR 630-BSA
ϵ [M ⁻¹ cm ⁻¹]	120 000	
λ_{\max} abs [nm]	621	645
λ_{\max} em [nm]	653	660
Counts per molecule [kHz]	19.9	147.4
Diffusion time [μ s]	53	279

Table 4: Properties of Fluorescent red 630 and 630-BSA measured at approx. 10⁻⁸ M, as reference Cy 5 had 89.3 kHz and diffusion time 54 μ s.

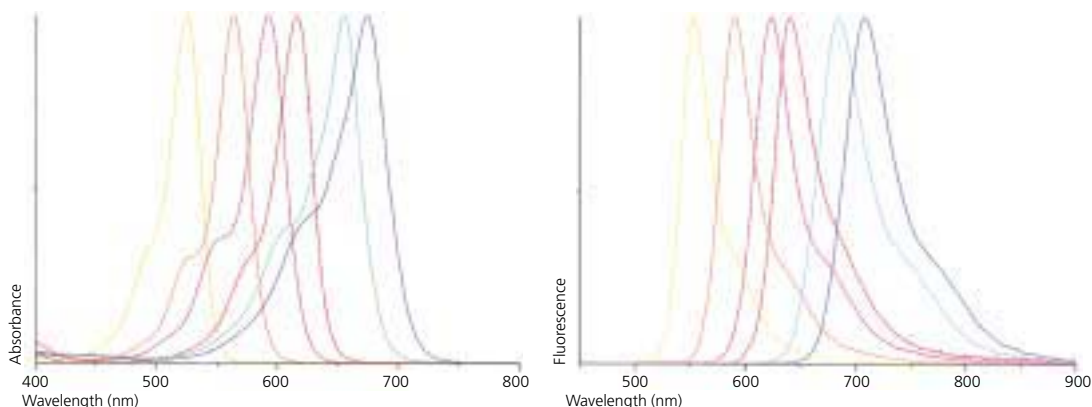


Figure 4: Absorption and fluorescence spectra of Atto labels measured in ethanol.

Luminescent Oxygen Probes for use in Sensing and Imaging

Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium dichloride ($\text{Ru}(\text{dpp})_3\text{Cl}_2$) and Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium hexafluorophosphate ($\text{Ru}(\text{dpp})_3(\text{PF}_6)_2$) have been widely used as probes for luminescent detection and quantitation of oxygen. The fluorescence of both dyes (abs. λ_{max} 455 nm, luminescence λ_{max} 613 nm) is strongly reduced by molecular oxygen due to dynamic quenching. Therefore, it is a viable oxygen probe based on either measurement of intensity or decay time. Quenching usually is expressed as the ratio of the decay times in the absence (τ_0) and presence (τ), respectively, of a quencher and can be presented as a Stern-Volmer plot as shown in figure 5. The graph says that, e.g., at a partial pressure of 200 hPa the decay time (and intensity) of $\text{Ru}(\text{dpp})_3(\text{PF}_6)_2$ has dropped by a factor of 2 due to dynamic quenching. $\text{Ru}(\text{dpp})_3\text{Cl}_2$ has been used for fiber optic sensors [5], studies on oxygen in skin and skin tumors [6, 7], measurement of oxygen flux through skin and for oxygen imaging [8]. $\text{Ru}(\text{dpp})_3(\text{PF}_6)_2$ proved to be superior for use in polystyrene membranes or beads. A protocol for embedding is given on our data sheet, available at www.sigma-aldrich.com/analytix.

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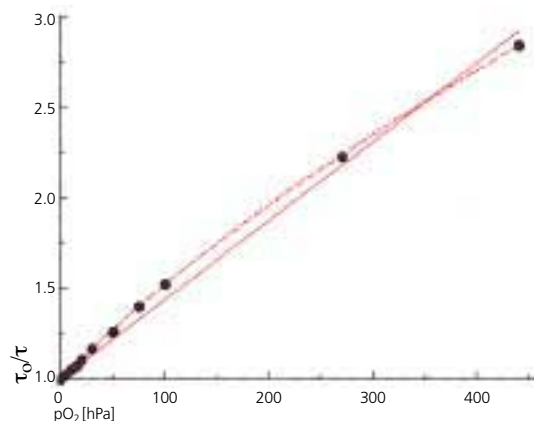


Figure 5: Quenching the fluorescence of $\text{Ru}(\text{dpp})_3(\text{PF}_6)_2$ by oxygen. At a partial pressure of 200 hPa the decay time (and intensity) has dropped by a factor of 2 due to dynamic quenching.

New $\text{Ca}^{2+}/\text{Mg}^{2+}$ Indicators with exceptional Brightness

In addition to the innovative Calcium indicators presented in the *Analytix* newsletter 2/2000 we are introducing new Calcium indicators with promising properties which provide an excellent addition to fluorescent $\text{Ca}^{2+}/\text{Mg}^{2+}$ indicators available already. Thio-H and Thio-OMe are low-affinity Ca^{2+} -indicators (K_d 55 μM , 126 μM). Such indicators are useful for determination of Ca^{2+} in intracellular compartments or for monitoring steep increases in intracellular concentration of Ca^{2+} . Compared to well-known low-affinity Ca^{2+} indicators, like Mag-fura-2, Thio-H is about three times brighter. This is due to a strong molar absorption as well as high quantum yields [9]. High fluorescent output allows low indicator concentrations to be used, which decreases the cation buffering effects of indicator [10]. Both indicators have the interesting property of being both excitation and emission (figure 6) ratiometric, thus ratiometric measurements are possible both in excitation and emission modes. Thio-OMe also shows a strong blue shift of emission maximum (485 nm to 418 nm) by binding to Ca^{2+} . For intracellular applications, AM-esters of both indicators have been prepared. Nonpolar AM-esters passively cross the plasma membrane and accumulate in the cytoplasm of living cells. After cleavage of the protecting groups by intracellular esterases, the polar fluorophore remains trapped within the cell.

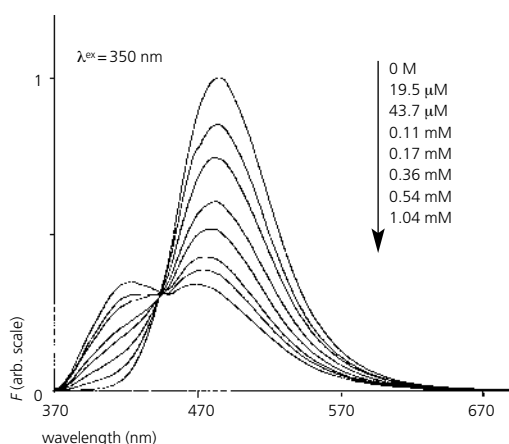


Figure 6: Thio-OMe, decrease of fluorescence emission at fluorescence maximum with increasing Ca^{2+} -concentration.

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