Recommended labeling protocols

Content

1. Introduction

Abberior offers a variety of fluorescent dyes with optimized properties for the labeling of biomolecules, spectroscopic studies, optical microscopy and particularly optical nanoscopy featuring superresolution. Along with non-switchable dyes (*Abberior* STAR series), we offer a series of caged (masked) fluorophores (*Abberior* CAGE series), as well as photoswitchable labels (*Abberior* FLIP series). Caged fluorescent dyes can be irreversibly photoactivated (uncaged) with UV- or visible light of 365-420 nm. Irradiation of the photosensitive FLIP-labels with light of 375 nm (one photon process) or 760 nm (two photon activation) results in the proton-assisted transition from the non-fluorescent spiro-amide isomer to the fluorescent "open-ring" form of the same amide. Detailed descriptions of the individual features and properties of Abberior dyes (including spectra, recommended wavelengths for excitation, STED and switching, etc.) are provided on the webpage.

In general, the *Abberior* labels offer very good properties regarding the traditional labeling requirements, photostability, water solubility, quantum efficiency, brightness, etc. As a consequence of being well qualified for STED applications, the labels are compatible with very high depleting light intensities, which are ~100 times larger than the excitation intensity. Thus, the *Abberior* STAR series is particularly suitable for conventional confocal microscopy. *Abberior* labels cover the visible spectral range from 530 nm to 660 nm.

The useful aminoreactive reagents are *N-hydroxysuccinimidyl* (NHS)-esters. This reactive group assists in forming a chemically stable bond between the label and the protein (antibody). For the labeling of thiol groups, the widely used reagents are maleimides, which form a stable thioether bond with the protein (antibody).

In the following are the proposed labeling protocols both for NHS and maleimide induced couplings of the label to the antibody. Additionally, *Abberior* offers a labeling protocol for the binding of the (secondary) antibody-coupled *Abberior* label with the target protein in the biological sample.

2. Labeling of antibodies/proteins with Abberior labels

The following section exemplifies the two conventional coupling techniques – NHS and maleimide induced coupling of an *Abberior* label with an antibody or other proteins.

Each section indicates required substances and materials. It describes the processes with the parameters to be controlled step by step.

2.1 Labeling with aminoreactive Abberior labels

Substances & Materials:

- *Abberior* NHS label (typically 0.2-0.4 mg)
- Antibody solution (typically 1-2 mg of protein, without BSA)
- Hydroxylamin (1.5 M in buffered solution of pH~8.3-8.5)
- DMF or DMSO and PBS (pH~6.5)
- $NaHCO_3$ (pH~8.3-8.5)
- Gel filtration column (e.g. Sephadex G25, PD-10 with a length of ~7 cm and diameter of ~1.7 cm)
- Bradford assay

See also graphical layout of the following protocol (fig 1).

Processes	**Parameters to be controlled:**
(A) Preparation of the dye solution	1. Dissolve dye in DMF or DMSO (10mg/ml)
	2. Use a 15-20-fold molar excess of the dye over the antibody
(A) Preparation of the antibody solution (1-2mg)	3. Add 1/10 of volume of 1 M aq. $NaHCO_3$ to provide pH = 8.0-8.5
(B) Mixing of antibody with *Abberior*-NHS label	4. Add slowly the calculated amount of Abberior dye solution to the magnetically stirred solution of the antibody with aq. $NaHCO_3$
	5. Gently stir at room temperature for 2 hours in the dark
	6. Add 20 μl 1.5 M NH_2OH (pH =8.0-8.5) to stop the reaction

Ⓒ Isolation of the dye-labeled antibody

7. Equilibrate the column with 30ml PBS buffer at pH~6.5

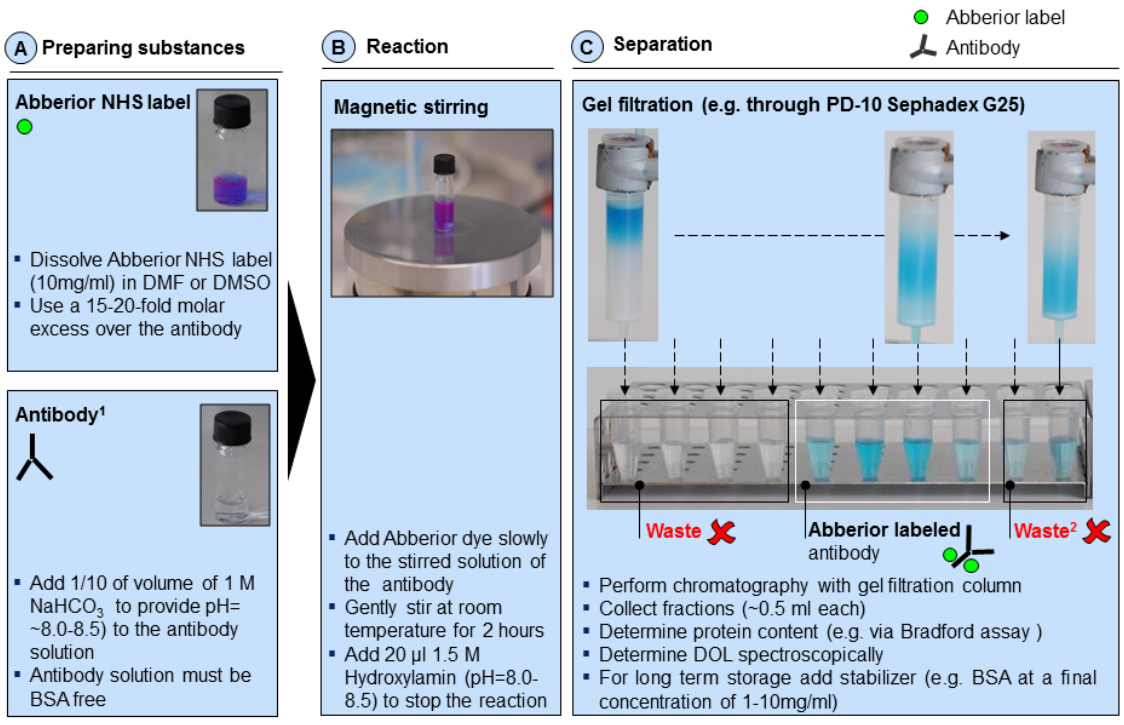
8. Transfer the solution with the labeled antibody on top of the gel filtration column and elute it with the PBS-buffer

9. Collect fractions (0.5 ml each) obtained in the course of gel filtration chromatography

10. Determine the antibody content in each fraction, e.g. via Bradford assay

11. Determine the DOL-value (see section 2.3)

Fig1. Abberior label - antibody conjugation (amine-reaction)



1 Alternatively other proteine with free amine group(s)
SOURCE: Abberior GmbH

2 Free dye (unbound to antibody)

| 1

Storage of the protein conjugate:

In general, the conjugate has to be stored under the same conditions as the unlabeled protein.

For storage in solution at 4°C, sodium azide (at final concentration of 2 mM) can be added as a preservative.

For long-term storage add stabilizers such as BSA (DOL needs to be determined before), separate the solution into aliquots and freeze at -20°C. Always protect from light and avoid repeated freezing and thawing.

2.2 Labeling with thiol-reactive Abberior labels (antibodies premodified with Traut's reagent)

Substances & Materials:

- *Abberior* maleimide label (typically 0.2-0.4 mg)
- Antibody (with thiol groups, typically 1-2 mg of protein, without BSA)
- DMF or DMSO and PBS (ph~6.5)
- Sodium phosphate, 0.15 M NaCl, 10mM EDTA ar pH~7.2-7.5
- 2-imionothiolane (Traut's reagent) dissolved in DMF
- Two gel filtration columns (e.g. Sephadex G25, PD-10 with a length of ~7 cm and diameter of ~1.7 cm)
- Bradford assay
- Hydroxylamin (1.5 M in buffered solution of pH~8.3-8.5)

See also graphical layout of the following protocol (fig 2).

Step I: Modification of the antibody with Traut's reagent

Traut's reagent creates the requisite sulfhydryls modification of the antibody necessary for conjugation with the maleimide-activated dye.

Processes	**Parameters to be controlled:**
Ⓐ Preparation of the antibody	1. Dissolve 1-2 mg/ml antibody in a 0.1 M phosphate buffer solution (pH= 7.2 -7.6) with 0.15 M NaCl and 10 mM EDTA (1-10 mg/ml)

Ⓐ Traut's regent

2. Stock solution of 2-imionothiolane in DMF (20-40-fold molar excess over antibody)

Ⓑ Mixing of antibody with Traut's reagent

3. Add solved Traut's regent to the antibody solution (not to exceed 10% of DMF in the final solution)

4. Gently stir at room temperature for 1 hour

Ⓒ Isolation of the bioconjugate

5. Equilibrate the column with 30ml PBS buffer at pH~6.5

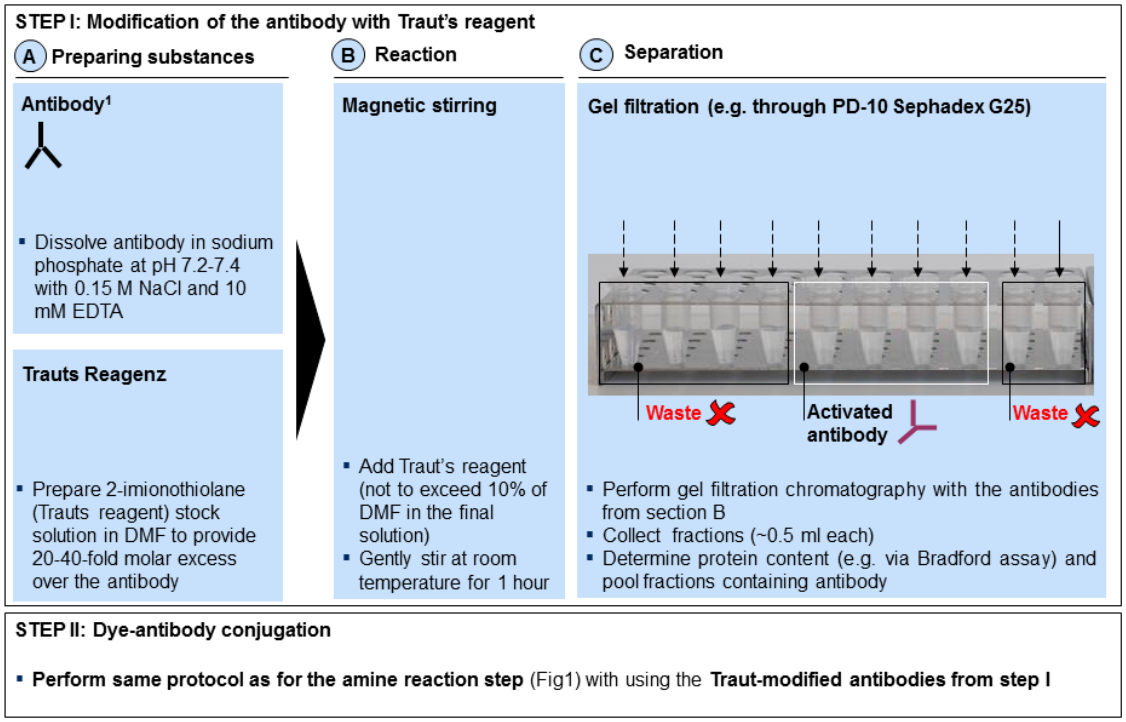
6. Transfer the solution with the labeled antibody on top of the gel filtration column and elute it with the PBS-buffer

7. Collect fractions (~0.5 ml each) obtained in the course of gel filtration chromatography

8. Determine antibody content (e.g. via Bradford assay) and pool fractions of containing similar concentrations of the antibody

Step II: Labeling of the Traut-modified antibody with the *Abberior* maleimide dye follows the protocol analogue to the NHS labeling in section 2.1.

Fig2. Abberior label-antibody conjugation (thiol-reaction)



1 Alternatively other protein without free thiol groups; in case of protein with free thiol groups directly proceed to step II
SOURCE: Abberior GmbH | 2

Storage of the proteine conjugate is performed under conditions similar to these described in section 2.1.

2.3 Determining the degree of labeling (DOL)

The degree of labeling (DOL) – the average number of dye molecules coupled to a protein molecule (i.e. the antibody) - can be determined by absorption spectroscopy utilizing the Lambert-Beer law. The required measurement of the UV to VIS spectrum of the conjugate solution obtained after gel filtration has to be done in a quartz (UV transparent) cuvette.

The equation below represents the DOL

$$DOL = \frac{A_{\max} / \varepsilon_{\max}}{A_{\Pr ot} / \varepsilon_{\Pr ot}} = \frac{A_{\max} \cdot \varepsilon_{\Pr ot}}{(A_{280} - A_{\max} \cdot C_{280}) \cdot \varepsilon_{\max}}$$

while C_{280} is a correction factor of the dye given by $C_{280} = \varepsilon_{280} / \varepsilon_{\max}$.

A denotes the absorbance of the dye at a given wavelength and is defined as $A = \varepsilon \cdot c \cdot d$ where ε denotes the extinction coefficient [$M^{-1} \cdot cm^{-1}$], c the molar concentration [mol/l] and d the path length of the light [dm]. All absorbance values A_x are measured as optical density units as [log I/I_0].

The following abbreviations were used:

Representing the label properties:

$A_{\max}$: absorbance of the label at the absorbance maximum

A_{280} : absorbance of the label at 280 nm

$\varepsilon_{\max}$: extinction coefficient of the label at the absorbance maximum

ε_{280} : extinction coefficient of the label at 280 nm

Representing the protein (i.e. antibody) properties:

$A_{\mathrm{Pr}ot}$: absorbance of the protein at 280 nm (absorption maximum of proteins)

$\varepsilon_{\mathrm{Pr}ot}$: extinction coefficient of protein at 280 nm

Note: The above equation is only accurate, if the extinction coefficient of the free dye $\varepsilon_{\max}$ at the maximum absorbance is equivalent to the value of the conjugate dye bond to the antibody (also at maximum absorbance). In practice the calculated values for the DOL can easily deviate by 20%.

3. Labeling of target proteins with antibody-coupled Abberior labels

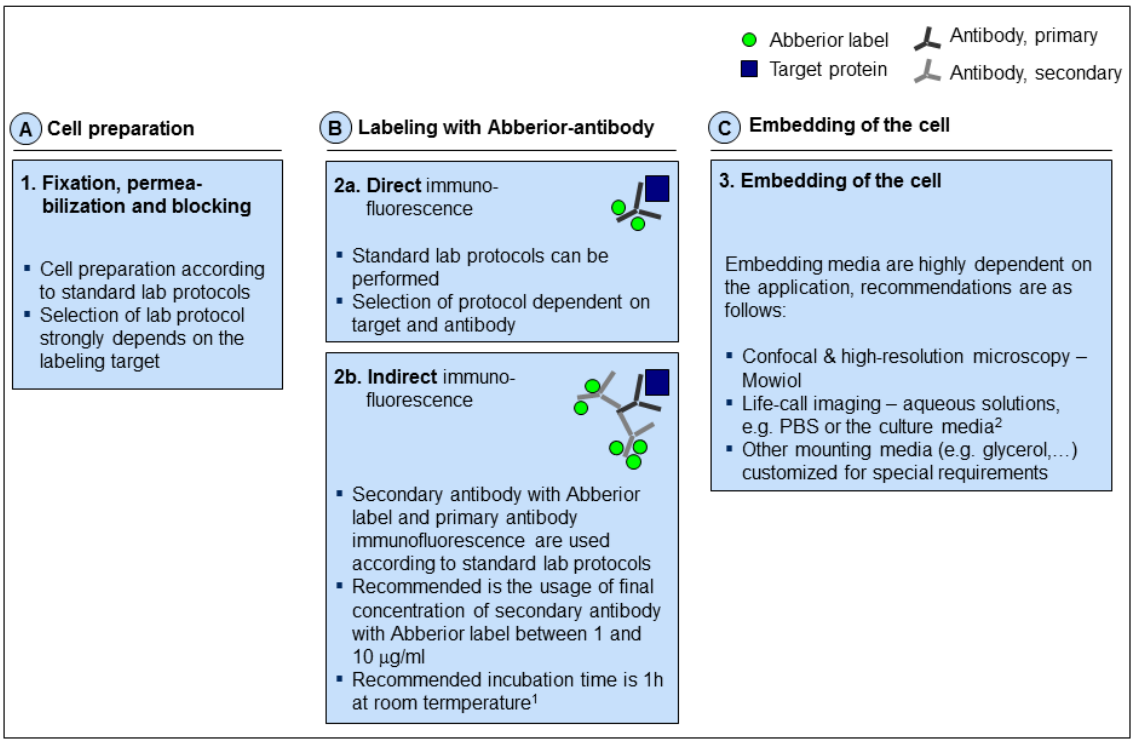
Note that in general the mounting media and the respective protocol are highly depending on the labeling target and the application. There does not exist a general protocol of embedding, in fact usually multiple labeling options exist. You can expect, that your current protocol can be used without adaptations if you use *Abberior* labeled antibodies. However, this section gives embedding recommendations on certain applications with using Abberior labels for high-resolution microscopy.

Substances & Materials:

- Chemicals for
    a. Fixation
    b. Permeabilization
    c. Blocking

    (no general recommendation possible, depends on the target of labeling)

Fig3. Cell labeling with Abberior-antibody labels



- **Direct** method: Labeling of the target with a primary antibody that is labeled with a *Abberior* label
- **Indirect** method: Target is recognized by a primary antibody; a secondary antibody is labeled with the *Abberior* label and recognizes specifically the primary antibody
- Mounting media (no general recommendation possible, depends on the imaging technique)

3.1 Abberior FLIP 565 – special recommendations for embedding

We recommend to mount the *Abberior* FLIP 565 labeled sample in Mowiol. This provides an environment with a high thermally induced (spontaneous) activation rate. Therefore only little UV light (approx. a few W/cm^2 in a widefield illumination mode) is required for sufficient switching of the compound. Illumination intensities of only a few kW/cm^2 are typical (for excitation at 532 nm).

4. Help and support

Abberior has extensive experience in labeling antibodies with *Abberior* labels. This knowledge we also offer as a service to our customers. Please refer to our support if you want us to label your antibody with designated *Abberior* labels (**support@abberior.com**).

Also in case of any questions regarding the labeling protocols, please contact us via the above given support email.