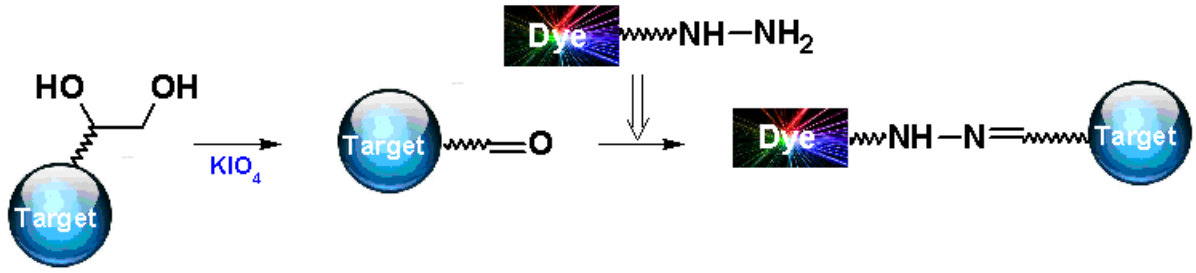
Labeling Cells and Glycoproteins with iFluor™ Dye Hydrazides

Introduction

iFluor™ dyes are a series of excellent fluorescent labeling dyes that span the full visible spectrum. All the iFluor™ dyes have excellent water solubility. Their hydrophilic property minimizes the use of organic solvents. The iFluor™ dyes also have much better labeling performance than the classic fluorescent labeling dyes such as FITC, TRITC, Texas Red®, Cy3®, Cy5® and Cy7®. Each iFluor™ dye was developed to match the spectral properties of a particular Alexa Fluor® or DyLight™ dyes. iFluor™ hydrazide dyes can be used for labeling cells. In addition, they are widely used to modify water-soluble biopolymers (such as proteins) through the formation of Schiff Base. These dyes are used for modifications of carbohydrates, glycoproteins and nucleic acids that are first periodate-oxidized to introduce aldehydes or ketones into the biopolymers for subsequent amination. The combination of periodate oxidation with amination provides an effective way for site-selective modifications of biopolymers.



Storage and Handling

Upon receipt, iFluor™ dyes should be stored at <-15 °C, and kept from light and moisture. The protein conjugate solution could be stored at 4 °C for two months without significant change when stored in the presence of 2 mM sodium azide and kept from light. For longer storage, the protein conjugates could be lyophilized or divided into single-used aliquots and stored at ≤ –60 °C, and protected from light.

Sample Neuron Cell Labeling Protocol

This sample labeling protocol was developed for labeling neuron cells with iFluor™ 488 hydrazide. You might need further optimization for your particular cells.

1. Prepare dye stock solution:

1.1 Dissolve 1 mg of iFluor™ 488 hydrazide in 100 µl of 200 mM KCl to make10 mM solution (10 mg/ml).

1.2 Sterilize the above solution by filtration.

2. Label Cells:

Label neuron cells (e.g., accessory planta retractor motoneurons in *Manduca sexta* larvae) with the iFluor™ 488 hydrazide stock solution (from Step 1) by passing hyperpolarizing current through the electrode.

3. Fix cells:

Fix the cells with 4% paraformaldehyde in 100 mM phosphate buffer (pH 7) for 30 min.

Note: Following fixation, the cells may be dehydrated in ethanol and cleared in xylene.

Sample Glycoprotein Labeling Protocol

This labeling protocol was developed for the conjugation of Goat anti-mouse IgG with iFluor™ 647 hydrazide. You might need further optimization for your particular proteins.

1. Prepare protein aldehyde solution (Solution A):

1.1 Dissolve 5 mg of goat anti-mouse IgG antibody in 0.5 mL of 0.1 M sodium acetate, 0.15 M NaCl, pH 5.5

Note: Make sure protein concentration >10 mg/ml for best labeling efficiency.

1.2 Treat the protein solution with 0.2 ml of 100 mM sodium periodate (21 mg/ml) by shaking for 30-60 min.

1.3 Stop the reactions by adding 30 µL ethylene glycol.

2. Prepare dye stock solution (Solution B):

Dissolve 1 mg iFluor™ dye hydrazide in 0.1 ml DMSO to make 10 mg/ml dye stock solution. Mix well by pipetting or vortex.

3. Run conjugation reaction:

3.1 Add the 10 µL of dye stock solution (Solution B) into 50 µL of the protein solution (Solution A) with effective shaking.

3.2 Continue to rotate or shake the reaction mixture at room temperature for 30-60 minutes in the dark.

4. Purify the conjugation

The following protocol is an example of dye-protein conjugate purification by using a Sephadex G-25 column.

4.1 Prepare Sephadex G-25 column according to the manufacture instruction.

4.2 Load the reaction mixture (directly from Step 3) to the top of the Sephadex G-25 column.

4.3 Add PBS (pH 7.2-7.4) as soon as the sample runs just below the top resin surface.

4.4 Add more PBS (pH 7.2-7.4) to the desired sample to complete the column purification. Combine the fractions that contain the desired dye-protein conjugate.

Note 1: For immediate use, the dye-protein conjugate might be aliquoted for multiple uses while for longer term storage, dye-protein conjugate solution need be concentrated or freeze dried.
Note 2: For small scale labeling, a ready-to-use spin commercial column is recommended for convenience.

Characterize the Desired Dye-Protein Conjugate

The Degree of Substitution (DOS) is the most important factor for characterizing dye-labeled protein. Proteins of lower DOS usually have weaker fluorescence intensity, but proteins of higher DOS tend to have reduced fluorescence too.

1. Measure absorption:

To measure the absorption spectrum of a dye-protein conjugate, it is recommended to keep the sample concentration in the range of 1-10 µM depending on the extinction coefficient of the dye.

2. Read OD (absorbance) at 280 nm and dye maximum absorption (λ_{max} = 649 nm for iFluor™ 647 dyes):

For most spectrophotometers, the sample (from the column fractions) need be diluted with de-ionized water so that the OD values are in the range of 0.1 to 0.9. The O.D. (absorbance) at 280 nm is the maximum absorption of protein while 649 nm is the maximum absorption of iFluor™ 647 hydrazide. To obtain accurate DOS, make sure that the conjugate is free of the non-conjugated dye.

3. Calculate DOS using the following equations:

3.1 Calculate protein concentration $[\text{Protein}] = \frac{\text{A280} - (\text{OD @ Dye Maximum Absoprtion X CF@280nm})}{\text{Protein Extinction Coefficient}} \times \text{dilution factor}$

3.2 Calculate dye concentration $[\text{Dye}] = \frac{\text{OD @ Maximum Absoprtion}}{\text{Dye Extinction Coefficient}} \times \text{dilution factor}$

3.3 Calculate the degree of labeling DOS = [Dye]/[Protein] $=[{}^{D}OD_{649} \times {}^{P}\varepsilon_{280}] / [250000 \times (A_{280} - 0.03A_{649})]$

[Dye] is the dye concentration, and can be readily calculated from the Bee-Lambert Law: $A=\varepsilon_{dye}CL$. [Protein] is the protein concentration. This value can be either estimated by the weight (added to the reaction) if the conjugation efficiency is high enough (preferably > 70%) or more accurately calculated by the Beer-Lambert Law: $A=\varepsilon_{protein}CL$. For example, IgG has the ε value to be 203,000 $cm^{-1}M^{-1}$. ${}^{P}\varepsilon_{280}$ = protein molar extinction coefficient at 280 nm (e. g. the molar extinction coefficient of IgG is 203,000

$cm^{-1}M^{-1}$). CF (dye absorption correction factor at 280 nm) = OD_{280}/OD_{649} = 0.03 for iFluor™ 647 dye hydrazide. 250,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of iFluor™ 647 hydrazide.

References

1. Hermanson GT (1996). *Biocojugate Techniques*, Academic Press, New York.
2. Haugland RP (1995). Coupling of monoclonal antibodies with fluorophores. *Methods Mol Biol* **45,** 205-21.
3. Brinkley M (1992). A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjug Chem* **3,** 2-13.

Appendix 1. iFluor™ Fluorescence-Labeling Dye Selection Guide Chart

iFluor™ Dye	Ex(nm)	Em (nm)	Features and Benefits	Ordering Information
***i*Fluor™ 350**	345 nm	442 nm	*Alternative to Alexa Fluor® 350 and DyLight™ 350* • Much stronger absorption • Much stronger fluorescence • Less environment-sensitive	#1020 (SE, NH_2-reactive) #1220 (labeling kit) #1050 (maleimide, SH-reactive)
***i*Fluor™ 405**	401 nm	420 nm	*Alternative to Cascade Blue®, Alexa Fluor® 405 and DyLight™ 405* • pH-insensitive fluorescence • Photostable	#1021 (SE, NH_2-reactive) #1051 (maleimide, SH-reactive)
***i*Fluor™ 488**	491 nm	514 nm	*Alternative to Alexa Fluor® 488 and DyLight™ 488* • pH-insensitive fluorescence • High labeling efficiency • Photostable	#1023 (SE, NH_2-reactive) #1052 (maleimide, SH-reactive) #1255 (labeling kit)
***i*Fluor™ 514**	518 nm	542 nm	*Alternative to Alexa Fluor® 514* • Strong fluorescence • Photostable	#1024 (SE, NH_2-reactive)
***i*Fluor™ 532**	542 nm	558 nm	*Alternative to Alexa Fluor® 532* • Strong fluorescence • Photostable	#1025 (SE, NH_2-reactive)
***i*Fluor™ 555**	555 nm	565 nm	*Alternative to Cy3®, Alexa Fluor® 555 and DyLight™ 550* • Strong fluorescence • More photostable than Cy3®	#1028 (SE, NH_2-reactive) #1053 (maleimide, SH-reactive) #1227 (labeling kit)
***i*Fluor™ 594**	594 nm	614 nm	*Alternative to Texas Red®, Texas Red-X, Alexa Fluor® 594 and DyLight™ 594* • Strong fluorescence • Photostable	#1029 (SE, NH_2-reactive) #1054 (maleimide, SH-reactive) #1230 (labeling kit)
***i*Fluor™ 610**	605 nm	627 nm	*Alternative to Alexa Fluor® 610* • Strong fluorescence • Photostable	#1038 (SE, NH_2-reactive)
***i*Fluor™ 633**	638 nm	655 nm	*Alternative to Alexa Fluor® 633* • Strong fluorescence • Photostable	#1030 (SE, NH_2-reactive) #1260 (labeling kit)
***i*Fluor™ 647**	649 nm	664 nm	*Alternative to Cy5®, Alexa Fluor® 647 and DyLight™ 650* • Strong fluorescence • More photostable than Cy5®	#1031 (SE, NH_2-reactive) #1055 (maleimide, SH-reactive) #1235 (labeling kit)
***i*Fluor™ 680**	676 nm	695 nm	*Alternative to Cy5.5®, IRDye® 700, Alexa Fluor® 680 and DyLight™ 680* • Strong fluorescence • More photostable than Cy5.5®	#1035 (SE, NH_2-reactive) #1056 (maleimide, SH-reactive) #1240 (labeling kit)
***i*Fluor™ 700**	685 nm	710 nm	*Alternative to Alexa Fluor® 700* • Strong fluorescence • Good photostability	#1036 (SE, NH_2-reactive) #1067 (maleimide, SH-reactive) #1245 (labeling kit)
***i*Fluor™ 750**	749 nm	776 nm	*Alternative to Alexa Fluor® 750 and DyLight™ 750* • Stronger fluorescence • More photostable than Cy7®	#1037 (SE, NH_2-reactive) #1058 (maleimide, SH-reactive) #1250 (labeling kit)
i*Fluor™ *A7	758 nm	784 nm	*Alternative to BD H7 and HiLyte Fluor™ 750* • Optimized for preparing APC tandem conjugate • More photostable than Cy7®	#1039 (SE, NH_2-reactive)
***i*Fluor™ 790**	782 nm	811 nm	*Alternative to IRDye® 800, Alexa Fluor® 790 and DyLight™ 800* • Stronger fluorescence • Higher Photostability	#1059 (maleimide, SH-reactive) #1265 (labeling kit) #1368 (SE, NH_2-reactive)

Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; Tel: 408-733-1055

Appendix 2. Spectral Properties of iFluor™ Fluorescent Labeling Dyes

Labeling Dye	Extinction Coefficient[1] ($cm^{-1}M^{-1}$)	Abs (nm)	Em (nm)	FQY[2]	CF at 260 nm[3]	CF at 280 nm[4]
iFluor™ 350	20,000	345	442	0.95	0.246	0.187
iFluor™ 405	29,000	401	420	0.91	0.229	0.697
iFluor™ 488	75,000	491	514	0.90	0.444	0.139
iFluor™ 514	80,000	518	542	0.95	0.316	0.182
iFluor™ 532	81,000	542	558	0.90	0.354	0.192
iFluor™ 555	150,000	555	565	0.10[5]	0.042	0.073
iFluor™ 594	110,000	594	614	0.15[5]	0.116	0.070
iFluor™ 610	90,000	605	627	0.85	0.468	0.441
iFluor™ 633	250,000	638	655	0.24	0.062	0.045
iFluor™ 647	250,000	649	665	0.25	0.026	0.030
iFluor™ 680	220,000	676	695	0.18	0.094	0.101
iFluor™ 700	220,000	685	710	0.20	0.040	0.036
iFluor™ 750	275,000	749	775	0.13	0.025	0.036
iFluor™ 790	250,000	782	811	0.09	0.098	0.091
iFluor™ A7	275,000	758	784	0.15	0.012	0.022

Note 1. Extinction Coefficient at their maximum absorption wavelength; 2. FQY = fluorescence quantum yield in aqueous buffer (pH 7.2); 3. CF at 260 nm is the correction factor used for eliminating the dye contribution to the absorbance at 260 nm (for oligo and nucleic acid labeling); 3. CF at 280 nm is the correction factor used for eliminating the dye contribution to the absorbance at 280 nm (for peptide and protein labeling); 5. Fluorescence intensity is significantly increased upon coupled to proteins.

Note 2. Texas Red® and Alexa Fluor® are the trademarks of Molecular Probes. CyDye, Cy3®, Cy5®, Cy5.5® and Cy7® are the trademarks of GE Health Care. HiLyte Fluor™ is tthe trademark of AnaSpec. IRDye® 700 and IRDye® 800 are the trademarks of Li-COR. iFluor™ is the trademark of AAT Bioquest.