1. Introduction

About HMSiR-NHS

HMSiR-NHS is a fluorescent probe for super-resolution imaging. This probe blinks spontaneously under the physiological conditions, without thiols, oxygen scavengers or irradiation of high-power laser, all of which were necessary for causing the blinking of fluorescent dyes in the previous dSTORM observation. Irradiation of 405 nm laser, which is needed for the observation with Alexa Fluor® 647, is not necessary with this probe. HMSiR-NHS (succinimidyl ester) forms covalent bonds with amino groups of antibodies or other proteins without condensing agent. HMSiR-NHS is applicable in the medium whose pH is neutral to weakly alkaline. This manual describes protocols for conjugating amine reactive compounds to about 5 mg of an IgG antibody.

2. Labeling protocol of IgG antibodies with HMSiR-NHS

Materials Required

- Anhydrous Dimethylsulfoxide (DMSO)
- Labeling buffer (0.1 M borate buffer pH 8.5, or other buffer (pH 7-9) which does not contain primary amines. Tris buffer is not compatible.)
- Washing buffer (PBS pH 7.4)
- Column for removal of dye: Gel filtration column of Sephadex G25 or ultrafiltration column (Nanosep® Centrifugal Devices Filter MWCO 10K Omega, PALL)

Procedure for labeling reaction

① Dissolve HMSiR-NHS in 18 μL DMSO to get 10 mM Dye solution.
② Dissolve IgG antibody in labeling buffer to get >1 mg/ml IgG buffer.
  ➤ Please don’t use PBS for this step to avoid deposition of dye.
③ Add Dye solution to the IgG buffer. Concentration of HMSiR-NHS should be 5 times greater than that of the IgG concentration. 3.3 μL of 10 mM Dye solution is appropriate for 1 mg of IgG.
④ Mix well and incubate for 30 min at 37°C.
⑤ Remove unbounded dye using appropriate column. Wash 10 times in case of ultrafiltration.
⑥ Collect the labeled antibody. The degree of dye (DOL) should be around 1.0-1.5.

Calculate the Degree of Labeling

By the purification method of the protocol above, the number of HMSiR-NHS molecules per an IgG antibody (degree of labeling: DOL) should be around 1.0~1.5, and the excess dye should be sufficiently removed. More accurate DOL can be calculated in the following way.
Measure the concentration of IgG antibodies by an appropriate method (Example: Pierce™ BCA Protein Assay Kit (Thermo Fisher Inc.)). Absorbance of HMSiR-NHS at pH 7.4 is low, but HMSiR-NHS takes open form in an acidic solution and shows stable and greater absorbance. Therefore, the concentration of the dye should be measured in pH 3.5 solution. First, create a standard absorption curve of HMSiR-NHS before labeling at maximum wavelength of 656 nm, by serially dilution with pH 3.5 citric acid buffer. Then, absorbance of the labeled protein at 656 nm in pH 3.5 is measured, and calculate concentration by approximate curve. ※ Please dilute HMSiR-NHS in pH 7.4 buffer with pH 3.5 citric acid buffer by 25 times or more in order to get stable absorbance from the dye in an acidic solution.

\[ \text{DOL} = \frac{M \text{ of HMSiR-NHS in labeled IgG antibody}}{M \text{ of labeled IgG antibody}} \]

![HMSiR-NHS Standard curve](image)

**Fluorescent observation**

Intensity of 647 nm laser for the evanescent field is 100 W/cm² by N-STORM (Nikon). 692/40 nm band pass emission filter (Semrock) is usable for the observation. Irradiation of 405 nm laser is not necessary. Observe cell in PBS without any additives. Capture 1000~5000 pictures as followed by the manual of the STORM.

**Storage**

Probes are packed under N₂ atmosphere, and are shipped at refrigerator state. After receipting, store under -20°C, desiccate and protect from light. We recommend using up DMSO solution of the dye.