Covalent labeling of CBD-EGFP with reactive fluorophores

For attachment of the dyes to cysteine, CBD-EGFP needs to be in 50 mM NaH_2PO_4 buffer, pH 7.5 at a protein concentrations of around 100 μM. A fresh solution of dye is prepared in pure DMSO. Add a small amount of dye to 30-40 μL DMSO and use this solution only that day. The exact concentration of the dye is determined spectrophotometrically by diluting the DMSO solution 1:5000 in methanol. The ISO-IAA dye extinction coefficient at maximum (approx. 600 nm) = 125,000. Usually we obtain concentrations around 20-25 mM for the original DMSO stock.

[ISO-IAA] = (OD_{600}*dilution factor) / 125000 (in mol/L)

A 300 μL aliquot of fresh CBD-EGFP protein (see protein preparation protocol) is transferred into a 2 ml eppendorf tube wrapped in foil (to protect from light). The dye is added in 2-3 aliquots to the CBD-EGFP solution to make the final dye : protein in the reaction about 5:1 or 6:1 (e.g. if the protein concentration is 100 μM, the dye concentration would be 600 μM). When adding dye, vortex gently, i.e. using a setting of 3 on the vortexer. The protein-dye mix is vortexed once briefly at higher setting to mix dye and protein completely. Using high dye to CBD-EGFP ratios (e. g. 10:1 or 15:1) in the reaction mixture can produce excessive amounts of precipitated material and "over-labeling" (dye:protein in the purified covalent adduct greater than 1). The optimal dye:protein ratio depends on the reactivity of the dye, so it can be different with other dyes. The tube is completely wrapped in foil and put for **1 hour** on a wheel at r. t. Here, the time is **critical**, since the dye is very reactive and longer labeling results in over-labeling. After the reaction is complete, 1 microliter β-ME is added to the reaction mix and the tube is incubated another 5-10 min while repeatedly inverted on a rotating wheel. This assures that there are no reactive species left during the following separation. This also may solubilize and remove non-covalently bound dye molecules from CBD-EGFP. The tube is centrifuged at rt to sediment insoluble material (e.g. for 2 min at 13,000 rpm in an Eppendorf benchtop microcentrifuge). The supernatant is then loaded on a small

G25 gel filtration column (0.5 cm x 6-8 cm) to separate the conjugate from free dye. This column is equilibrated and run in 50 mM NaH_2PO_4 buffer, pH 7.5. The first colored band to elute contains CBD-EGFP conjugated to dye. Fractions of approximately 200 µL are collected (approximately 4 drops. An aliquot (3-5 µL) of each fraction is analyzed by 12% SDS-PAGE to confirm the presence of the modified CBD-EGFP and the purity of the sample. Fluorescence visualization of the gel assures labeling with the dye.

Determination of the labeling efficiency (dye : protein ratio)

This can be difficult and will be approximate. For accurate measurements, at least until you obtain consistent readings, it is a good idea to use both methods described here and compare their results.

Protein concentration:

Protein concentration can be determined by taking an absorbance spectrum of the conjugate solution. Dilute a small aliquot (5-10 µL) in 50mM Tris HCl, pH 7.5-8.0 and use OD280 for the calculation:

$$[\text{CBD-EGFP}] = (OD_{280} * \text{dilution factor}) / 28260 \text{ (in mol/L)}$$

However, the protein absorbance at 280 overlaps dye absorbtion. One can determine the protein concentration independently by comparing a sample of the labeled material to unlabeled CBD-EGFP using at different sample concentrations. Colorimetric assays, limited to those with readouts that do not overlap dye absorbance, have been more variable in our hands.

Dye concentration:

The dye concentration is determined by taking an absorbance spectrum of the protein conjugate. A small aliquot of the conjugate (5-10 µL) is diluted in DMSO. This solvent will overwhelm effects of the protein on the dye absorbance, to produce a

consistent dye extinction coefficient. OD at the dye absorbance maximum (approximately 600) is used to calculate the dye concentration.

$$[Dye] = (OD*dilution\ factor) / 125000\ (in\ mol/L)$$

Usually, about 40-60% of the CBD-EGFP is recovered as purified conjugate, due to some precipitation during labeling and loss during gel filtration. The final elute concentration is usually 50-60 μM. Labeling efficiency under these conditions varies between 0.7-0.9 dye: protein. Labeled CBD-EGFP solution is aliquoted into 15-20 μL aliquots, flash frozen with dry ice or liquid nitrogen and stored at –80°C. Alternatively, the CBD-EGFP-dye conjugate may be kept without freezing at 4°C for about one week.

Peri – I used Lou’s d:p values here.