

# A new protocol for fluorescent quantitative labeling of individual proteins for live cell internalization assays

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## Background

A single protein can be chemically labeled with different fluorescent dyes in order to get insights about its cellular localization and molecular mechanism of action [1]. Overall, an ideal fluorescent dye should be specific and stable, and should not interfere with the physicochemical and conformational properties of the protein. For internalization assays in living cells, organic fluorophores such as fluorescein (FITC) and rhodamine (TRITC) are the dyes of choice since they have a short lifetime (<5 ns), and they are brighter and more photostable than other fluorescent labels [2]. They also have a small size, thus interfering less with the biological system (i.e. protein). The main disadvantages of these compounds are their low specificity and unknown binding stoichiometry. In this work, we have tailored a minimal labeling protocol, originally developed for proteomic applications, in order to tag single proteins for being used in live cell internalization assays.

## Methods

The original 2D-DIGE minimal labeling protocol ensures that only 1–2% of the available Lys is labeled. Thus, this protocol facilitates successful labeling of an individual protein at a specific location ( $\epsilon$  amino group of Lys) and with a well-defined stoichiometry (1:1). Moreover, this modification only adds just about 500 Da to the protein total molecular weight, thus minimizing perturbations of the physicochemical and conformational properties of the protein. Overall, the full standard protocol is accomplished in 4-5 hours and comprises three different steps (Figure 1). Firstly, the protein of interest is resuspended in the reconstitution buffer and labeled with the CyDye fluorophore of choice. After incubation, labeling is stopped by adding an excess of L-lysine (Lys) to the reaction mixture. Secondly, the excess of free Lys-bound CyDye is removed to minimize background in the internalization experiments. At this point, the Cy-labeled protein can be concentrated if necessary, by filtration through a size exclusion membrane of suitable cutoff. Finally, in the last step, the quality of the labeling reaction is tested by SDS-PAGE.

## Results

To validate experimentally our protocol, we carried out the fluorescent labeling of IBB1, a major soybean protease isoinhibitor of the Bowman-Birk family that is currently being investigated as colorectal chemopreventive agent [3], with two different CyDye fluors. A single protein band of about 12 kDa was visible after scanning the polyacrylamide gel at 488 nm (IBB1-Cy2) or 635 nm (IBB1-Cy5), respectively, proving the success of the minimal labeling reaction. Then, we monitored the *in vivo* internalization dynamics of the labeled IBB1 protein in human colorectal adenocarcinoma HT29 cells. We observed that the CyDye-labeled IBB1 proteins crossed the plasma membrane of HT29 cells after a few minutes and was gradually accumulated, forming fluorescent patches randomly distributed across the cytoplasm. We also tested the suitability of this method for carrying out multiplex experiments. Thus, two different combinations of labels were tested. Firstly, IBB1-Cy2 internalization was combined with nuclei staining using Hoechst 33342 dye. The fluorescent signal of IBB1 did not overlap with Hoechst-specific labeling, indicating that the protein did not internalize into the cell nucleus. Secondly, IBB1-Cy2 internalization was combined with the fluorescent probe FM 4-

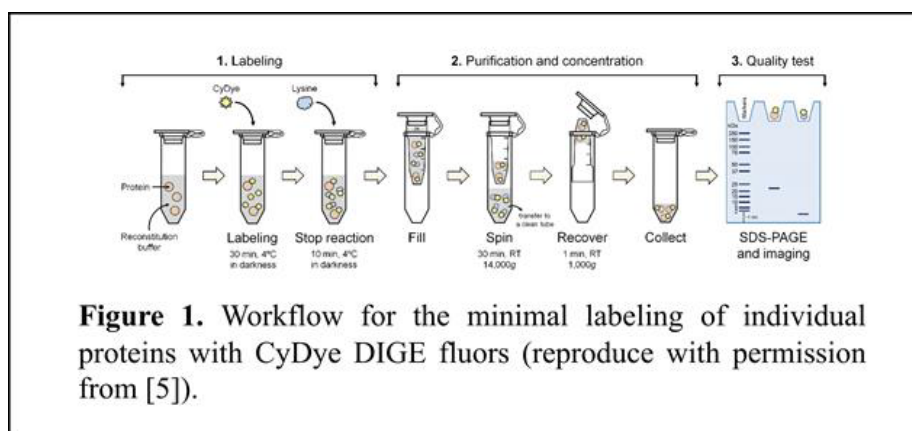
64, an endocytic tracer [4]. We observed that the fluorescent signal from the endosome marker colocalized with the Cy2-labeled IBB1 protein in the cytoplasm of HT29 cells, suggesting that IBB1 is internalized through one of the existing endocytosis pathways.

### Conclusions

In this work, we have adapted a protocol that was originally developed for two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) applications, to label proteins with CyDye fluors for single-molecule internalization assays in living cells. This protocol is suitable for both proteins purified from their natural sources and recombinant proteins expressed in heterologous systems (e.g. *E. coli*, *P. pastoris*, etc.). This “minimal labeling” method offers a number of advantages including specificity and known stoichiometry, simplicity, high reproducibility, and sensitivity and allows multiplexing while minimizing perturbations of the biological system. Moreover, the minimal labeling protocol is suitable for multiplex experiments, either combining up to three different proteins labeled with Cy2, Cy3, and Cy5, respectively, or using CyDye fluors together with other fluorescent labels. Finally, since only a single lysine (Lys) residue per protein molecule is labeled, this method is also quantitative.

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### Graphic:



### Keywords:

CyDye-fluors, IBB1, internalization-assays, minimal fluorescent-labeling

### Reference:

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