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Chapter

A Protocol for Minimal Single Protein Labeling with CyDye Fluors for Live Cell Internalization Assays

Antonio J. Castro, Alfonso Clemente and Juan de Dios Alché

Abstract

Individual proteins chemically labeled with fluorescent dyes can be localized and tracked in real-time experiments in order to get insights about the site and molecular mechanism of action. Here, 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 "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, since only a single lysine (Lys) residue per protein molecule is labeled, this method is also quantitative. 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. Then, we analyzed the *in vivo* internalization dynamics of the labeled IBB1 protein in human colorectal adenocarcinoma HT29 cells.

Keywords: Bowman-Birk inhibitors, CyDye fluors, fluorescence microscopy, HT29 cells, IBB1, live cell internalization assay, minimal fluorescent labeling

1. Introduction

Fluorescence-based assays allow protein quantification and measuring protein interactions, enzymatic activity, and conformational changes. Individual proteins chemically labeled with fluorescent dyes can also be localized and tracked in real-time experiments throughout the cell in order to get insights about the site and molecular mechanisms of their action [1]. The choice of the fluorescent marker will largely depend on the nature of the downstream assay to be performed with the labeled protein. Overall, an ideal fluorescent label should be specific, bright, and stable and should not interfere with the physicochemical and conformational properties of the protein. Unfortunately, according to our knowledge, none of the existing fluorescent tags accomplish all these criteria.

To analyze the dynamics of labeled proteins throughout the cell, we should combine live cell imaging and single-molecule methods. For this purpose, different

fluorescent tags and labeling methods are available, including genetically encoded fluorescent proteins (e.g., GFP) [2], direct labeling with organic fluorophores (e.g., Cy3) [3], tag-mediated peptide and protein labeling methods (e.g., SNAP-tag) [4], and quantum dots (Qdots) [5]. For live cell internalization assays, organic fluorophores are preferable since they have a short lifetime (<5 ns) and they are brighter and more photostable than other fluorescent labels [6]. They also have a small size, thus interfering less with the biological system. Fluorescein (FITC) and rhodamine (TRITC) dyes have been often used for this purpose [7, 8]. The main disadvantages of these organic fluorophores are the low specificity of the labeling reaction and the difficulty of defining its stoichiometry. Additionally, the unbound dye could interfere with subsequent applications, so it should be removed before performing downstream procedures.

Cyanines are synthetic fluorescent dyes belonging to polymethine group that yield brighter and more stable fluorescence than FITC and TRITC [9]. At protein level, cyanines have been used to track single proteins in live cell assays [10], as well as in molecular interaction studies by fluorescence resonance energy transfer (FRET) [11]. Moreover, cyanine dyes constitute the basis of the two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) technology [12]. This method was developed to multiplex different protein samples and minimize gel-to-gel quantitative variations. CyDye DIGE fluors are available as minimal and saturation labeling dyes. The former are used when the amount of sample is not a limiting factor, while saturation labeling dyes are intended for 2D-DIGE applications with scarce biological samples (e.g., those collected by laser capture microdissection). Minimal dyes can also be used for specific labeling of cell-surface proteins prior to 2D-DIGE analysis [13]. In this paper, we have tailored the 2D-DIGE minimal labeling protocol in order to tag single proteins for being used in live cell internalization assays. This protocol is suitable for both proteins purified from their natural sources and recombinant proteins expressed in heterologous systems (e.g., *Escherichia coli*, *Pichia pastoris,* etc.). Moreover, this method overcomes the problems of specificity and stoichiometry of the labeling reaction. To validate experimentally our protocol, we carried out internalization assays of IBB1, a major soybean protease isoinhibitor of the Bowman-Birk family, which is currently being investigated as colorectal chemopreventive agent [14, 15].

2. Description and experimental design

2D-DIGE CyDye minimal labeling dyes have an NHS ester reactive group that specifically binds to the ε amino group of lysine (Lys) residues by an amide linkage. Lysine represents about 3.6 ± 0.6% and 7.0 ± 0.6% of the total amino acid content in plant and animal proteins, respectively [16]. Accordingly, a general labeling procedure would likely result in labeling most of Lys residues. However, the 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 (ε amino group of Lys) and with a well-defined stoichiometry (one Lys per protein molecule, 1:1). Moreover, this modification only adds just about 500 Da to the protein total molecular weight, thus minimizing perturbations of the biological system.

Overall, the protocol 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. This phase ends when the labeling is stopped by adding an excess of L-lysine (Lys) to the reaction mixture. When carrying out multiplex experiments with other fluorescent labels, it is important to select the appropriate CyDye fluors in order to visualize independently each fluorescent signal when imaging. Secondly,

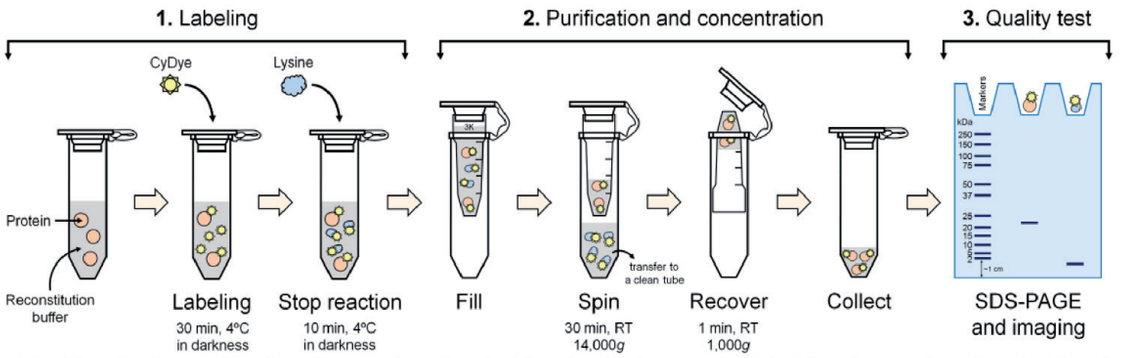

Figure 1.
Workflow for the minimal labeling of individual proteins with CyDye DIGE fluors.

the excess of free CyDye and exchange buffers is removed for sample cleanup. At this point, it is also possible to concentrate the Cy-labeled protein if necessary, by filtration through a size exclusion membrane of suitable cutoff. Finally, in the last step, the quality of the labeling reaction is verified by SDS-PAGE. The full standard protocol, which is accomplished in just a few hours, is described in detail below.

3. Labware chemicals and instruments

3.1 Reagents and solutions

Prepare all solutions using ultrapure water (18 MΩ cm at 25°C) and analytical grade reagents:

- 99.8% anhydrous N,N-dimethylformamide (DMF).
- Amersham™ CyDye™ DIGE Fluor minimal labeling kit (catalog no. 25-8010-65, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Alternatively, individual Cy™2, Cy™3, and Cy™5 minimal dyes are also available (see **Note 1**).
- 1× reconstitution buffer: 30 mM Tris-HCl (pH 8.5), 7 M urea, 2 M thiourea, and 4% (w/v) CHAPS (see **Note 2**).
- L-Lysine monohydrochloride.
- pH test strips, pH 4.5–10.0, resolution: 0.5 pH units.
- pH adjustment solution: 1× reconstitution buffer at pH 9.5 (see **Note 2**). Alternatively, 50 mM sodium hydroxide can be used.
- 12% polyacrylamide precast (or handmade) mini gels, 10-well, 30 μL.
- Prestained protein standards, broad range (e.g., Precision Plus Protein™ Dual Xtra, catalog no. 1610377, Bio-Rad).
- 2× gel loading buffer: 120 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, and 120 mM DTT (see **Note 3**).
- 1× electrophoresis running buffer: 25 mM Tris-HCl, 192 mM glycine, and 0.2% (w/v) SDS.

3.2 Materials and instruments

- Protein low-binding microcentrifuge 1.5 mL tubes.
- Set of variable volume (0.5–10, 10–100, and 100–1000 μL) single-channel pipettes.
- Amicon® Ultra-0.5 mL, Ultracel® 3 K filter device (catalog no. UFC500308. Millipore Corp., Burlington, MA, USA).
- Refrigerated centrifuge equipped with a fixed-angle rotor that can accommodate microcentrifuge 1.5 mL tubes, rated for 14,000 *g*.
- Vortex mixer.
- Mini-PROTEAN®-type vertical electrophoresis system.
- Image scanner equipped with a standard set of lasers (488, 532, and 635 nm) and emission filters.

4. Protein minimal fluorescent labeling procedure

4.1 Reconstitution of CyDye fluors

CyDye fluors are delivered lyophilized in opaque, conical microcentrifuge 1.5 mL tubes, so they have to be reconstituted before its use as follows:

a. Remove the CyDye from the freezer and leave for 5 min at room temperature.

b. Spin the microcentrifuge tube at 12,000 *g* for 10 s to collect the dye at the bottom.

c. Reconstitute the CyDye by adding the appropriate volume of DMF to obtain a stock solution at a final concentration of 1 mM. For example, in the case of a 5 nmol size pack, add 5 μl of DMF (see **Note 4**).

d. Replace the cap and vortex vigorously for 30 s.

e. Spin the microcentrifuge tube for 30 s at 12,000 *g*.

f. The reconstituted CyDye is now ready for protein labeling (see **Note 5**).

4.2 Minimal fluorescent labeling

Proceed as quickly as possible in order to minimize the time of handling, and also avoid exposure to light, which can quench the fluorescent properties of CyDye fluors. In addition, keep protein samples on ice to minimize degradation by proteases, and always use protein low-binding plastic microcentrifuge tubes as many proteins are able to bind to glassware:

a. Prepare a CyDye working solution to a final concentration of 400 μM by adding one volume of CyDye stock solution to 1.5 volumes of DMF.

b. Resuspend the lyophilized protein in the reconstitution buffer to a final concentration of 1 μg/μL.

c. Check that the pH remains at values near to 8.5 by spotting 1 μL on a pH indicator strip (see **Note 6**).

d. Take an aliquot of protein sample, and mix with the suitable volume of CyDye working solution. For optimal labeling, use 400 pmol of CyDye per 50 μg of protein (see **Note 7**).

e. Mix well by vortexing and centrifuge at 14,000 *g* for 10 s.

f. Incubate the mixture on ice for 30 min in the dark.

g. Add 1 μl of 10 mM L-lysine, mix by pipetting, and incubate for 10 min to stop the labeling reaction.

4.3 Purification and concentration of CyDye-labeled proteins

After labeling, the remaining dye (i.e., the CyDye bound to free L-Lys) should be removed from the protein solution before proceeding with downstream internalization experiments. Optionally, the labeled protein can be also concentrated at this step if necessary:

a. Place the Amicon Ultra-0.5 mL 3 K filter device into a microcentrifuge tube (see **Note 8**).

b. Add up to 500 μL of CyDye-labeled protein sample to the filter device.

c. Centrifuge at 14,000 *g* for 30 min. The final volume and concentration factor of the concentrate will depend on the spin time (see **Table 1** from [17]).

d. Transfer the first filtrate to a clean 1.5 mL polypropylene tube, and store at −20°C in the dark until use (see **Note 9**).

e. Dilute the concentrate with the desired cell culture medium to a final volume of 500 μL (see **Note 10**).

f. Spin at 14,000 *g* for 10–30 min as above and discard the filtrate.

g. Repeat Steps 5–6 thrice.

Dye	Color	Excitation max. (nm)	Emission max. (nm)	Laser (nm)	Emission filter
Cy2	Green	489	506	[1,2]Blue (488)	[1]520BP40; [2]530nmBP
Cy3	Red	550	570	[1,2]Green (532)	[1]580BP30; [2]605nmBP
Cy5	Blue	649	670	[1,2]Red (635)	[1]670BP30; [2]695nmBP

[1]For Amersham™ Typhoon™ 9400 imager (GE Healthcare Bio-Sciences).
[2]For PharosFX™ Molecular© Imager (Bio-Rad).

Table 1.
Spectral properties of CyDye™ fluors and lasers and emission filters used to scan fluorescent gels.

h. To recover the concentrated protein, place the filter device upside down in a new clean 1.5 mL tube supplied by the manufacturer.

i. Spin for 2 min at 1000 *g* to transfer the concentrated protein sample from the filter device to the tube (see **Note 11**).

4.4 Quality control of the labeling reaction

Before its use in internalization experiments, it is important to check that the protein was correctly labeled and purified. For this purpose, we will run a small sample of the labeled protein on 1-D SDS-PAGE along with a purity control sample as follows:

a. Make a 12% SDS-PAGE gel following standard protocols [18]. Alternatively, an equivalent precast gel can be used.

b. Mix a volume of the CyDye-labeled protein solution (~2 μg) with an equal volume of 2× gel loading buffer into a clean 1.5 mL tube by vigorously pipetting. Do not exceed the maximum capacity (30 μL) of the gel well.

c. Heat the protein sample at 95°C for 5 min to ensure full reduction of the labeled protein.

d. Make serial dilutions of the purity control sample (see **Section 4.3, d**) by adding different volumes (e.g., 5, 10, and 15 μL) to equal volumes of 2× gel loading buffer. It is not necessary to heat these samples.

e. Load the labeled protein sample along with the purity control samples in successive lanes on the gel.

f. Add the prestained protein standards according to the manufacturer's instructions at both ends of the gel, in order to track the electrophoretic migration of samples.

g. Run the samples at 180 V until the 2 kDa marker has nearly reached the bottom of the gel (leave about 1 cm).

h. Scan the gel at 100 μm resolution using the appropriate laser and emission filter (**Table 1**).

5. Experimental validation of the protocol

To validate experimentally our protocol, we carried out live cell internalization assays of IBB1, a major soybean protease isoinhibitor of the Bowman-Birk family with an ability to inhibit both trypsin- and chymotrypsin-like enzymes. This protein was purified from a commercially available BBI mixture of soybean isoinhibitors and tagged with CyDye fluors as described above. Then, its internalization in human colorectal adenocarcinoma HT29 cells was monitored using fluorescence microscopy.

5.1 Purification of soybean IBB1 protease inhibitor

A major Bowman-Birk isoinhibitor from soybean, called IBB1, was isolated from a commercial sample containing a mixture of IBB1 and IBBD2, as well as other inhibitors from the Kunitz family (catalog no. T9777, Sigma-Aldrich), as previously described [19]. Briefly, the commercial extract was resuspended to a final concentration of 0.7 mg/mL in 10 mL of elution buffer (25 mM sodium acetate buffer, pH 4.4). Aliquots of 2 mL were filtered through a Minisart® 0.2 μm pore size syringe filter (catalog no. 17821 K, Sartorius, Gottingen, Germany) and loaded on a Mono S 5/50 GL cation exchange column (catalog no. 17516801, GE Healthcare Bio-Sciences) connected to an ÄKTA™ FPLC apparatus (GE Healthcare Bio-Sciences). Fractionation was carried out using a linear gradient of 0–0.22 M NaCl in 25 mM sodium acetate buffer (pH 4.4) at a flow rate of 1 mL/min. The elution was monitored at 280 nm, and 0.5 mL fractions were collected.

In order to identify that fraction containing the pure IBB1 protein, trypsin (TIA) inhibitory activity of eluted samples was spectrophotometrically assayed in 96-well flat-bottom microtiter plates by using N_{α}-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA, catalog no. B4875, Sigma-Aldrich) as specific substrate [19]. Chymotrypsin inhibitory activity (CIA) evaluation was measured using a modified small-scale quantitative assay with N-benzoyl-L-tyrosine ethyl ester (BTEE, catalog no. B6125, Sigma-Aldrich) as previously described [20, 21]. The purity and identity of the IBB1 fraction containing both TIA and CIA were further confirmed by SDS-PAGE denaturing electrophoresis and peptide mass fingerprinting (PMF) analysis, following standard protocols. Finally, the IBB1 sample was dialyzed extensively against distilled water during 48 h at 4°C using a Spectra/Por™ 3, MWCO 3.5 kD, dialysis membrane (catalog no. 734-0687, Spectrum™ Labs, New Brighton, MN, USA), quantified using the standard BCA assay [22], and freeze-dried in a LyoQuest-55 lyophilizer (catalog no. 61644, Telstar, Terrassa, Spain) until use.

5.2 Minimal labeling of IBB1 protein

Two IBB1 samples were labeled with CyDye fluors Cy2 and Cy5, respectively, using the minimal labeling protocol described above. After purification and concentration steps by filtration, each labeled IBB1 protein was suspended in ~50 μL of Gibco® DMEM culture medium (catalog no. A1443001, Thermo Fisher Scientific, Waltham, MA, USA). To check the quality of labeling, approximately 2 μg of Cy2- and Cy5-labeled IBB1 proteins was analyzed by SDS-PAGE on a 12% polyacrylamide gel along with the control samples (i.e., Lys-$_{Cy2}$ and Lys-$_{Cy5}$) according to standard procedures and visualized in a PharosFX™ Molecular© Imager (Bio-Rad) using the appropriate laser and emission filter for each fluorochrome (see **Table 1**).

A single protein band with an apparent molecular weight of about 12 kDa was visible after scanning the gel at 488 nm (IBB1-$_{Cy2}$) and 635 nm (IBB1-$_{Cy5}$), proving the success of the minimal labeling reaction (**Figure 2**). When the gel was scanned at 488 nm, the fluorescent signal from IBB1-Cy5 was not detected. The signal from IBB1-Cy2 was also abolished when the gel was scanned at 635 nm, thus confirming the specificity of the labeling reaction (data not shown). Moreover, no fluorescent signal corresponding to the Lys-$_{Cy2}$ and Lys-$_{Cy5}$ bands were detected in IBB1 samples as compared with control samples (**Figure 2**), indicating that labeled IBB1 samples were pure and, thus, suitable for being used in live cell internalization experiments.

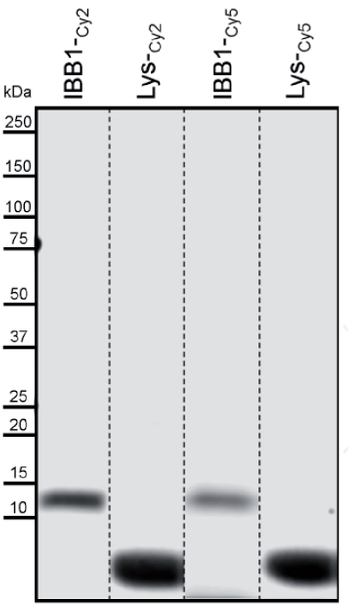

Figure 2.
Quality control by SDS-PAGE of IBB1 labeling with Cy2 and Cy5 dyes. Protein standards (kDa) are displayed on the left.

5.3 Live cell internalization experiments

5.3.1 IBB1 internalization assays

Human colorectal adenocarcinoma HT29 cells were cultured in DMEM medium, supplemented with 5% (v/v) fetal bovine serum, 2 mM glutamine, and 1% (v/v) antibiotic-antimycotic solution (catalog no. A5955, Sigma-Aldrich), as previously described [23]. After trypsinization, chambered μ-slides with a glass coverslip bottom (catalog no. 80444, Ibidi, Gräfelfing, Germany) were inoculated at a density of 25,000 HT29 cells per well in 700 μL of DMEM culture medium and incubated at 37°C under 5% CO2 in humidified air for 24 h to allow the cells to adhere to the chamber bottom. The culture medium was then supplemented with 50 μg of Cy5-labeled IBB1. Cell internalization dynamics was monitored using a C1 confocal laser microscope (Nikon Corp., Tokyo, Japan). Z-series images of HT29 cells were recorded at different time (min) intervals by exciting the sample with a red diode (633 nm) and processed with the software EZ-C1 Gold v2.10 build 240 (Nikon). Three independent experiments were carried out.

At $t_{0\ min}$, no fluorescent signal was observed inside HT29 cells (data not shown). The Cy5-labeled IBB1 protein crossed the plasma membrane of HT29 cells after a few minutes and was gradually accumulated, forming fluorescent patches randomly distributed across the cytoplasm (**Figure 3**). Negative controls of HT29 cells cultured with the unlabelled IBB1 protein did not show any fluorescent signal (data not shown).

5.3.2 Multiplex experiments

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. Here, two different combinations of labels were tested. Firstly, nuclei of colon cancer cells were stained by adding to the culture medium 1 μL of Hoechst 33342 dye solution (catalog no. H1399, Thermo Fisher Scientific). HT29 cells were incubated for

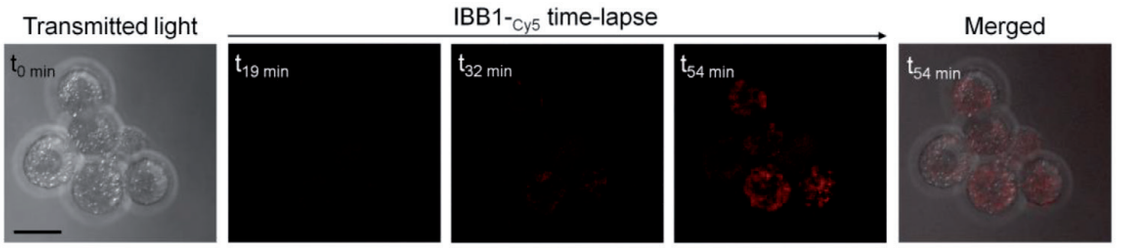

Figure 3.
Internalization dynamics of IBB1 protein into human colorectal adenocarcinoma HT29 cells. Fifty μg of IBB1-$_{Cy5}$ was added to the culture medium at the onset of culture, and HT29 cells were imaged at different time intervals. Cy5 fluorescence images are reproduced with permission from [24]. Bar = 50 μm.

30 min, and the culture medium was then supplemented with IBB1-$_{Cy2}$. Secondly, in addition to Hoechst, the fluorescent probe FM 4-64 (catalog no. T3166, Thermo Fisher Scientific), an endocytic tracer [25], was added to the growth medium (final concentration = 5 μg/mL) at the same time with IBB1-$_{Cy2}$. After 90 min of culture, the medium was supplemented with 0.35 μL of 50 mM brefeldin A (BFA), a potent inhibitor of exocytosis [26], and incubated for 30 min before imaging.

Cell internalization dynamics of IBB1-$_{Cy2}$ was monitored using an Eclipse Ti-U microscope (Nikon Corp.) equipped with a *p*E-300^{white} illumination system (CoolLED Ltd., Andover, UK) and a DFK 72AUCO2 camera (The Imaging Source, Bremen, Germany). Hoechst, Cy2, and FM 4-64 fluorescence images were obtained using a UV, blue, and green LED light, respectively. Images were merged with the Image-Pro Plus v6.0 software (Media Cybernetics, Rockville, MD, USA). For each combination of fluorescent labels, three independent experiments were carried out.

After the first 3 hours of culture, HT29 cells showed a very intense green fluorescent labeling from IBB1, forming patches randomly distributed across the polarized cytoplasm (green fluorescence image in **Figure 4**). However, the fluorescent signal of IBB1 did not overlap with Hoechst-specific labeling (blue fluorescence image corresponding to DNA in **Figure 4**), indicating that the protein did not internalize into the cell nucleus after this period of time.

On the other hand, we observed that the fluorescent signal from the endosome marker FM 4-64 (red fluorescence image in **Figure 5**) was localized in the plasma membrane and the cytoplasm of HT29 cells after 120 min of culture as expected. This signal clearly overlapped with labeling from IBB1 in the cytoplasm (green fluorescence image in **Figure 5**), giving a yellowish fluorescence in the merged image. There was no overlapping between nuclei (blue fluorescence image in **Figure 5**) and either IBB1 or FM4-64 labeling. These results suggest that IBB1 is internalized into the cytoplasm of HT29 cells through one of the existing endocytosis pathways.

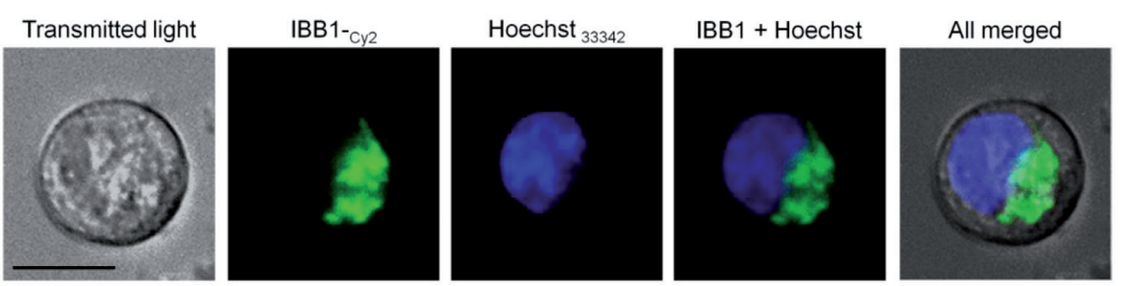

Figure 4.
Internalization of IBB1 (green) into the cytoplasm of human colorectal cancer HT29 cells. Nuclei were stained with Hoechst 33342 (blue). HT29 cells were imaged after 180 min of culture. Bar = 10 μm.

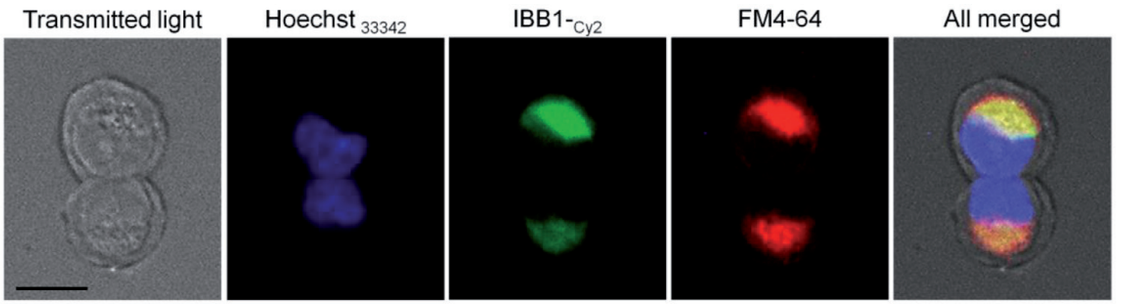

Figure 5.
Mechanism of IBB1 (green) internalization into human colorectal cancer HT29 cells. Nuclei were stained with Hoechst 33342 (blue). The endocytic tracer FM 4-64 (red) was added to the culture medium at the onset of the experiment. HT29 cells were imaged after 120 min of culture. Bar = 10 μm.

6. Troubleshooting guide

Users of the minimal fluorescent labeling protocol might encounter several problems, whose potential causes and their corresponding remedies are summarized in **Table 2**.

Probable causes	Remedies
Problem #1: low sample recovery in the concentrate	
1a. Adsorption of the protein to the filter device surfaces	1a. Remove the concentrate immediately after centrifugation
1b. Protein precipitation due to sample overload on the filter device	1b. Dilute the sample in the reconstitution buffer to a final concentration of 1 μL/mL before centrifugal spin
Problem #2: the Cy-labeled protein is not detected on the 1-D gel	
2a. DMF of poor quality	2a. Only use 99.8% anhydrous DMF
2b. DMF has been opened for longer than 3 months	2b. DMF must be less than 3 months old from day of opening
2c. The lifetime of the reconstituted CyDye has been exceeded	2c. Once reconstituted, the CyDye is stable and usable until the expiry date or for 2 months, whichever is sooner
2d. The CyDye has been exposed to light or left out of the freezer for a long time	2d. Make ready-to-use aliquots, and always store them at −20°C in the dark until use
2e. The pH in the labeling mixture has dropped below 8.0	2e. Adjust the pH to 8.5 by adding small volumes of the pH adjustment buffer (**Note 6**)
2 f. The protein solution concentration is less than 1 mg/mL	2 f. Concentrate the protein solution using a suitable filter device, and check again the pH and concentration of the sample
2 g. Incorrect CyDye to protein ratio	2 g. For optimal labeling, use 400 pmol of CyDye per 50 μg of protein
2 h. Insufficient protein sample loading on the gel	2 h. It is recommended to load between 1 and 5 μg of protein
Problem #3: detection of more than one fluorescent bands in the labeled protein sample on the 1-D gel	
3a. More than one Lys residue per molecule has been labeled with the dye (ladder effect)	3a. Decrease CyDye to protein ratio until only a single band is visible
3b. Presence of multimeric forms of the protein due to insufficient reduction	3b. Increase the concentration of DTT in the gel loading buffer
3c. Presence of protein fragments due to proteolysis	3c. Minimize protease activity by keeping protein samples at 4°C throughout the protocol
Problem #4: presence of free dye in the protein sample	
4a. Insufficient purification of the labeled protein	4a. Increase the number of filtration cycles in the protocol

Table 2.
Minimal fluorescent labeling with CyDye fluors of single proteins: troubleshooting guide.

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Conflict of interest

The authors have no competing interests to declare.

Notes

Note 1. **Caution!** CyDye fluors are harmful in contact with the skin and if inhaled or swallowed. It is strongly recommended to wear lab gloves, mask, and coat when handling them to avoid staining clothing and the skin.

Note 2. Make aliquots and store them at −20°C until use.

Note 3. Important! Do not add bromophenol blue to the gel loading buffer. This compound is visible when scanning the gel at 635 nm, so it can interfere when using Cy5.

Note 4. Important! DMF must be less than 3 months old from day of opening. DMF is degraded over time, forming amine compounds that will compete with the protein for the CyDye.

Note 5. Important! This stock solution must be stored at −20°C and in the dark, being stable and usable until the expiry date or for 2 months, whichever is sooner.

Note 6. Critical step! There is little or no fluorescent labeling when pH values drop below 8.0. If pH of the protein solution has fallen to values below 8.0 after adding the lysis buffer, adjust the pH to 8.5 by adding a few drops of pH adjustment solution.

Note 7. Labeling reactions can be scaled up using more protein and CyDye flour.

Note 8. The Ultracel membrane contains trace amounts of glycerin. If this compound interferes with upstream analysis, pre-rinse the filter device with ultrapure water. If interference continues, rinse with 0.1 N NaOH followed by a second spin of ultrapure water. **Caution!** Do not allow the membrane to dry out once wet.

Note 9. This first filtrate contains the remaining CyDye, bound to free Lys, to be used for quality control of the labeling reaction (Section 4.4).

Note 10. The lysis buffer contains chemical reagents that are toxic for cells (e.g., urea, CHAPS, etc.). For its removal, add the same culture medium used for growing cells in your internalization assays.

Note 11. Alternatively, labeled protein samples can be stored for at least 3 months at −80°C in the dark.

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References

[1] Toseland CP. Fluorescent labeling and modification of proteins. Journal of Chemical Biology. 2013;**6**:85-95. DOI: 10.1007/s12154-013-0094-5

[2] Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science. 1994;**263**(5148): 802-805. DOI: 10.1126/science.8303295

[3] Puljung MC, Zagotta WN. Labeling of specific cysteines in proteins using reversible metal protection. Biophysical Journal. 2011;**100**(10):2513-2521. DOI: 10.1016/j.bpj.2011.03.063

[4] Hinner MJ, Johnsson K. How to obtain labeled proteins and what to do with them. Current Opinion in Biotechnology. 2010;**21**:766-776. DOI: 10.1016/j.copbio.2010.09.011

[5] Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, et al. Quantum dots for live cells, in vivo imaging, and diagnostics. Science. 2005;**307**(5709): 538-544. DOI: 10.1126/science.1104274

[6] Ha T, Tinnefeld P. Photophysics of fluorescent probes for single-molecule biophysics and super-resolution imaging. Annual Review of Physical Chemistry. 2012;**63**:595-617. DOI: 10.1146/annurev-physchem-032210-103340

[7] Maxfield FR, Schlessinger J, Shecter Y, Pastan I, Willingham MC. Collection of insulin, EGF, and α2-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. Cell. 1978;**14**:805-810. DOI: 10.1016/0092-8674(78)90336-7

[8] Yavelow J, Scott CB, Mayer TC. Fluorescent visualization of binding and internalization of the anti-carcinogenic Bowman-Birk type protease inhibitors in transformed fibroblasts. Cancer Research. 1987;**47**:1602-1607

[9] Ernst LA, Gupta RK, Mujumdar RB, Waggoner AS. Cyanine dye labeling reagents for sulfhydryl groups. Cytometry. 1989;**10**(1):3-10. DOI: 10.1002/cyto.990100103

[10] Okamoto Y, Ninomiya H, Miwa S, Mas T. Cholesterol oxidation switches the internalization pathway of endothelin receptor type A from caveolae to clathrin-coated pits in Chinese hamster ovary cells. Journal of Biological Chemistry. 2000;**275**:6439-6446. DOI: 10.1074/jbc.275.9.6439

[11] Meyer BH, Martinez KL, Segura JM, Pascoal P, Hovius R, George N, et al. Covalent labeling of cell-surface proteins for in-vivo FRET studies. FEBS Letters. 2006;**580**:1654-1658. DOI: 10.1016/j.febslet.2006.02.007

[12] Unlü M, Morgan ME, Minden JS. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. Electrophoresis. 1997;**18**(11):2071-2077. DOI: 10.1002/elps.115018113

[13] Hagner-McWhirter A, Winkvist M, Bourin S, Marouga R. Selective labelling of cell-surface proteins using CyDye DIGE Fluor minimal dyes. Journal of Visualized Experiments. 2008;**21**. DOI: 10.3791/945

[14] Clemente A, Marín-Manzano MC, Jiménez E, Arques MC, Domoney C. The anti-proliferative effects of TI1B, a major Bowman-Birk isoinhibitor from pea (*Pisum sativum* L.), on HT29 colon cancer cells are mediated through protease inhibition. British Journal of Nutrition. 2012;**108**:S135-S144. DOI: 10.1017/S000711451200075X

[15] Utrilla MP, Peinado MJ, Ruiz R, Rodríguez-Nogales A, Algieri F, Rodríguez-Cabezas ME, et al. Pea (*Pisum sativum* L.) seed albumin extracts show anti-inflammatory effect

in the DSS model of mouse colitis. Molecular Nutrition & Food Research. 2015;**59**:807-819. DOI: doi.org/10.1002/mnfr.201400630

[16] Gorissen SHM, Crombag JJR, Senden JMG, Waterval WAH, Bierau J, Verdijk LB, et al. Protein content and amino acid composition of commercially available plant-based protein isolates. Amino Acids. 2018;**50**:1685-1695. DOI: 10.1007/s00726-018-2640-5

[17] Amicon® Ultra-0.5 Centrifugal Filter Devices: User Guide. Burlington, MA, USA: Millipore Corporation; 2011

[18] Gallagher SR. SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Current Protocols Essential Laboratory Techniques. 2012;**6**:7.3.1-7.3.28. DOI: 10.1002/9780470089941.et0703s06

[19] Clemente A, Moreno FJ, Marín-Manzano MC, Jiménez E, Domoney C. The cytotoxic effect of Bowman-Birk isoinhibitors, IBB1 and IBBD2, from soybean (*Glycine max*) on HT29 human colorectal cancer cells is related to their intrinsic ability to inhibit serine proteases. Molecular Nutrition & Food Research. 2010;**54**:396-405. DOI: 10.1002/mnfr.200900122

[20] Clemente A, Jiménez E, Marín-Manzano MC, Rubio LA. Active Bowman-Birk inhibitors survive gastrointestinal digestion at the terminal ileum of pigs fed chickpea-based diets. Journal of the Science of Food and Agriculture. 2008;**88**:523-531. DOI: 10.1002/jsfa.3115

[21] Clemente A, MacKenzie DA, Jeenes DJ, Domoney C. The effect of variation within inhibitory domains on the activity of pea protease inhibitors from the Bowman-Birk class. Protein Expression and Purification. 2004;**36**:106-114. DOI: 10.1016/j.pep.2004.03.015

[22] Walker JM. The bicinchoninic acid (BCA) assay for protein quantitation. In: Walker JM, editor. The Protein Protocols Handbook. 2nd ed. Totowa, NJ: Humana Press; 2009. pp. 11-15. DOI: 10.1007/978-1-59745-198-7_3

[23] Clemente A, Gee JM, Johnson IT, Mackenzie DA, Domoney C. Pea (*Pisum sativum* L.) protease inhibitors from the Bowman-Birk class influence the growth of human colorectal adenocarcinoma HT29 cells in vitro. Journal of Agricultural and Food Chemistry. 2005;**53**:8979-8986. DOI: 10.1021/jf051528w

[24] Soria M, Porras P, Moya FJ, Sánchez N, Rodríguez B, Ruiz ML, et al. Internalization of the anti-carcinogenic IBB1, a major Bowman-Birk isoinhibitor from soybean (*Glycine max*), in HT29 colon cancer cells. igh School Students for Agricultural Science Research. 2014;**3**:59-68

[25] Bolte S, Talbot C, Boutte Y, Catrice C, Read ND, Satiat-Jeunemaitre B. FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. Journal of Microscopy. 2004;**214**:159-173. DOI: 10.1111/j.0022-2720.2004.01348.x

[26] Helms JB, Rothman JE. Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. Nature. 1992;**360**(6402):352-354. DOI: 10.1038/360352a0