**TITLE:**

**Proteome-wide Quantification of Labeling Homogeneity at the Single Molecule Level**

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# **KEYWORDS:**

single molecule imaging, proteomics, fluorescence labeling, gel electrophoresis, labeling homogeneity, cell lysate

# **SUMMARY:**

Here, we present a protocol to assess the labeling homogeneity for each protein species in a complex protein sample at the single molecule level.

# **ABSTRACT:**

Cell proteomes are often characterized using electrophoresis assays, where all species of proteins in the cells are non-specifically labeled with a fluorescent dye and are spotted by a photodetector following their separation. Single molecule fluorescence imaging can provide ultrasensitive protein detection with its ability for visualizing individual fluorescent molecules. However, the application of this powerful imaging method to electrophoresis assays is hampered by the lack of ways to characterize the homogeneity of fluorescent labeling of each protein species across the proteome. Here, we developed a method to evaluate the labeling homogeneity across the proteome based on a single molecule fluorescence imaging assay. In our measurement using a HeLa cell sample, the proportion of proteins labeled with at least one dye, which we termed ‘labeling occupancy’ (LO), was determined to range from 50% to 90%, supporting the high potential of the application of single molecule imaging to sensitive and precise proteome analysis.

# **INTRODUCTION:**

Proteome analysis, which aims to quantify the entire set of protein molecules expressed in the cell, is a valuable approach in current biological and medicinal studies. This analysis commonly relies on mass spectrometry, which identifies protein species based on spectra generated through protein ionization[1–3](https://paperpile.com/c/2NQckN/6DdU+Zd1b+R15c). An alternative method for proteome analysis is electrophoresis, including polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis and two-dimensional (2D) gel electrophoresis. This method relies on non-specific fluorescent labeling of all the protein molecules in the analyzed cells, followed by electrophoretic separation and detection and quantification of each protein species. To achieve the required non-specific protein labeling, one strategy is to use fluorescent dyes that can bind to proteins via electrostatic and hydrophobic interactions, such as Coomassie Blue and Sypro-Ruby[4–6](https://paperpile.com/c/2NQckN/pq4q+srCr+WIge). An alternative strategy is to use covalent labeling with dyes containing N-hydroxysuccinimide (NHS) ester or maleimide, which can covalently bind to proteins through common residues such as primary amines and thiols, respectively[7,8](https://paperpile.com/c/2NQckN/VmWGA+6w91C).

Meanwhile, the sensitivity of fluorescence detection is ideal for analyzing low-abundance proteins and small numbers of cells. Single molecule fluorescence imaging is one of the most sensitive methods that allows the detection of individual fluorescent dyes and labeled proteins in vitro and in vivo[9–15](https://paperpile.com/c/2NQckN/Y9i0A+6Hd0N+LLUyU+oMhxp+kM4Km+ozZKj+tKBDV). Application of this imaging method to electrophoresis-based proteome analysis is expected to enable highly sensitive and quantitative assays by counting individual fluorescently-labeled proteins. However, it remains unclear whether labeling with fluorescent dyes is homogeneous enough across all the protein molecules, and how this homogeneity is affected by different protein species (**Figure 1**). Simple bulk solution measurements can be used to obtain a molar ratio of fluorescent dyes to proteins called ‘coupling efficiency’[8](https://paperpile.com/c/2NQckN/6w91C) or ‘labeling efficiency’, but this property does not provide information on the homogeneity of the labeling among protein molecules.

Here, we describe the protocol for an assay to investigate the labeling homogeneity for all protein species in the cell (**Figure 2**)[16](https://paperpile.com/c/2NQckN/YUzCx). The two key steps of this assay are protein purification and imaging. In the first step, all the proteins in the cells are fluorescently labeled and biotinylated, then extracted separately using gel electrophoresis followed by electroelution. In the second step, fluorescence properties of individual protein molecules in the extracted samples are evaluated based on single molecule imaging. From this data, parameters important for the counting analysis, such as the percentage of proteins labeled with a least one dye, which we call labeling occupancy (LO)[16](https://paperpile.com/c/2NQckN/YUzCx), and the average number of fluorescent dyes bound to a single protein molecule (*n̄*dye), can be characterized. In the protocol, an optimized procedure for labeling the HeLa cell proteome with NHS ester-based Cyanine 3 (Cy3) dye is presented as an example, and can be modified with other labeling procedures according to desired research goals.

# **PROTOCOL:**

## Cell preparation

* 1. Cultivate HeLa cells in a 10 cm dish at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.
  2. Collect exponentially growing cells once they have reached 70% confluency, following ATCC instructions[17](https://paperpile.com/c/2NQckN/heoB).
     1. Rinse cells with 5 mL of 1x phosphate buffered saline (PBS) (pH 7.4). Remove PBS.
     2. Add 1 mL of 0.1% trypsin-ethylenediaminetetraacetic acid (EDTA). Incubate 5 min at 37 °C.
     3. Monitor the progression of cell dissociation by microscopy.
     4. Once cells become round and are detached from the dish, add 4 mL of growth medium, and break cell agglomeration by pipetting.
     5. Count the number of cells using a cell counter.
     6. Centrifuge at 150 x *g* for 10 min in a centrifuge tube. Remove the medium and resuspend the cell pellet with 5 mL of 1x PBS (pH 7.4).
     7. Repeat step 1.2.6. Resuspend cells in 1x PBS (pH 7.4) to a final concentration of 106 cells/mL using the count number in step 1.2.5.

NOTE: The cell suspension can be aliquoted and stored at -80 °C for several months. Repeating a freeze/thaw cycle should be avoided.

## Cell lysis and fluorescent labeling

* 1. Make 10 mL of lysing buffer (0.1 M borate, 1% sodium dodecyl sulfate (SDS) and 1% Tween 20). Adjust to pH 12 using 2 M NaOH.

CAUTION: Concentrated NaOH is corrosive. Wear suitable gloves and glasses when preparing this solution.

NOTE: Lysing buffer can be stored for up to one week at room temperature.

* 1. Mix 100 μL of lysing buffer with 1 μL of 1 M dithiothreitol (DTT) and 4 μL of 50% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS).
  2. Mix 1 μL of the cell culture (approximately 1,000 cells) with the mixed lysing buffer from step 2.2. Homogenize the solution by slowly pipetting to avoid bubbles. Incubate in the dark at room temperature for 5 min with gentle agitation. Re-homogenize the solution by pipetting slowly.
  3. Add 1 μL of 2 μg/mL Cy3 NHS-ester dye. Homogenize the solution by slowly pipetting to avoid bubbles. Incubate for 5 min in the dark at room temperature with gentle agitation.

NOTE: The high pH of this buffer causes neutralization of lysine residues in proteins, leading to a higher reactivity.

* 1. Repeat the addition of 1 μL of 2 μg/mL Cy3 NHS-ester dye. Homogenize the solution by slowly pipetting to avoid bubbles. Incubate for 10 min in the dark at room temperature with gentle agitation.

NOTE: This repeated addition of NHS-ester dye can increase the labeling efficiency because some of the Cy3 NHS-ester dye is hydrolyzed and deactivated by the high pH of the reaction buffer.

* 1. Adjust to pH 7.2 by adding 100 μL of 0.8 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH pH 7.2 to quench the reaction. Homogenize the solution by slowly pipetting to avoid bubbles.
  2. Add 20 μL of 19 mg/mL biotin-(polyethylene glycol (PEG))2-amine and 5 μL of 20 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Homogenize the solution by slowly pipetting to avoid bubbles. Incubate for 1 h in the dark at room temperature with gentle agitation.

NOTE: This biotinylation step is necessary to fix the protein molecules on a microscope coverslip at a fixed density and with no bias for protein species.

* 1. Remove unreacted labeling reagents and concentrate the sample using an ultrafiltration column with 10 kDa cut-off, following the manufacturer's instructions.

NOTE: The protocol can be paused here. The sample can be stored at -80 °C for several weeks.

## Proteome separation and recovery

* 1. Add 4x SDS sample buffer (200 mM Tris-HCl pH 6.8, 4% SDS, 4% glycerol and 0.4% bromophenol blue).

NOTE: To avoid dye damage, the protein sample should not be heated as done in standard SDS-PAGE, and should be kept at room temperature.

* 1. Perform standard SDS-PAGE for the protein sample and a molecular ladder, using 20% acrylamide gel. Migrate the sample in the gel at a constant voltage (160 V) until the migration front just exits the gel.
  2. Image the gel to identify the location of the protein bands (**Figure 3**).
  3. Cut out the gel portions including protein fractions of interest using a sharp blade, based on the band locations. Fractioning at 16, 23, 55 and 120 kDa peaks, and at 19–25, 25–32, 32–42, 42–54, 54–69, 69–97, 97–136 and 136–226 kDa regions are shown in **Figure 3**.
  4. Extract proteins using a dialyzer by electroelution with a pore size of 6–8 kDa, following the manufacturer's instructions.

NOTE: The extracted sample can be stored at -80 °C for several weeks.

## Microscope coverslip preparation

* 1. Prepare 200 μL of avidin buffer (5 mM HEPES-NaOH pH 7.2, 10 ng/mL avidin and 2 mg/mL bovine serum albumin (BSA)) for each sample.
  2. Prepare coverslips of 22 mm x 22 mm and 0.15 mm thickness. Expose the coverslips to air plasma using a plasma cleaner for 1 min to clean and activate their surfaces.
  3. Coat the coverslips with avidin by spin-coating 200 μL of avidin buffer using a spin coater for 5 seconds at 500 rpm, followed by 30 seconds at 1,000 rpm. Incubate the coverslips for 15 min at room temperature to let them dry.
  4. Prepare coverslips for the protein sample.
     1. Dilute the protein sample (from 3.5) 100 times in 5 mM HEPES-NaOH pH 7.2.
     2. Place 100 μL of the diluted sample on the surface at the center of the avidin-coated coverslip (from step 4.3).
     3. Incubate in the dark for 15 min at room temperature.
  5. Prepare the positive control.
     1. Place 100 μL of 10 ng/mL purified fluorescent biotin in 5 mM HEPES-NaOH pH 7.2 at the center of the avidin coated coverslip (from step 4.3).
     2. Incubate in the dark for 15 min at room temperature.

NOTE: This positive control coverslip provides the density of avidin binding spots, which is used to calculate the LO.

* 1. Prepare the negative control.
     1. Spin-coat the plasma cleaned coverslip with 200 μL of 5 mM HEPES-NaOH and 2 mg/mL BSA (without avidin).
     2. Incubate for 15 min at room temperature to dry.
     3. Add 100 μL of 10 ng/mL purified fluorescent biotin in 5 mM HEPES-NaOH pH 7.2.
     4. Incubate in the dark for 15 min at room temperature.

NOTE: This negative control coverslip provides the amount of non-specific binding of fluorescent biotin to the coverslip without avidin.

* 1. Rinse each coverslip with 200 μL of distilled water by pipetting at the edges of the coverslip so as not to touch the middle of the coverslip with the tip. Repeat this wash three times.

NOTE: After aspirating water on the coverslip, water should be immediately placed back on the coverslip to prevent it from drying out completely.

* 1. Expose air plasma to the same number of coverslips as in step 4.2.
  2. Place the cleaned coverslip on the sample-bound coverslip from step 4.7 to avoid drying.

## Observation with single molecule fluorescence microscopy

* 1. Start up a wide-field or evanescent field fluorescence microscope.

CAUTION: Direct or scattered laser radiation can cause eye or skin damage. Wear suitable protective goggles and shield the laser light path.

NOTE: For the microscope setup, please refer to our previous publication[16](https://paperpile.com/c/2NQckN/YUzCx). Briefly, a wide-field or evanescent field fluorescence microscope equipped with a high-power 488 nm laser excitation, a high numerical aperture objective lens and a high sensitivity camera can be used for this measurement. Further, for an automated measurement, a computer-controlled XY translational sample stage, mechanical shutters for laser excitations and an auto-focusing system are recommended to be installed.

* 1. Set the coverslip on the microscope and find the focus.
  2. Perform a tile scan to obtain at least 100 images.

NOTE: The laser illumination should be controlled with mechanical shutters to be exposed to the sample only when recording images with the camera, to minimize photobleaching. If there are many samples, automated measurement using a computer program to control the microscope devices is recommended. Each image typically includes 100–500 single molecule spots when using a 60x objective lens.

## Image analysis and extraction of information

* 1. If the image has unevenness due to the laser illumination pattern, carry out image processing to correct it[18](https://paperpile.com/c/2NQckN/4sx9b).
     1. Acquire a reference image by measuring with a coverslip sample consisting of uniformly-spread dyes using a camera setting the same as in step 5.3.
     2. Acquire an offset image by measuring with no laser excitation using the same camera setting.
     3. Subtract each pixel value in every sample image and the reference image with the value of the offset image.
     4. Divide each pixel value in the subtracted sample images by the value in the subtracted reference image.
  2. Collect properly acquired images and remove the image background.
     1. Remove images containing large fluorescent aggregates manually.
     2. Remove the image background by applying the rolling-ball algorithm[19](https://paperpile.com/c/2NQckN/L9Qmi) with a ball radius of 50 pixels using ImageJ[20](https://paperpile.com/c/2NQckN/OAfp).
     3. Sharpen images by subtracting blurred images using ImageJ (sharpen image function).
  3. Find and analyze single molecule spots in images.
     1. Identify single molecule spots with the Yen threshold algorithm[21](https://paperpile.com/c/2NQckN/M8U0q) using ImageJ.
     2. Filter spots with less than two pixels to exclude dark noise of the camera and spots more than 20 pixels to exclude aggregations of proteins using ImageJ (ROI manager).
     3. Count the number of spots in each image. Using the illumination corrected images (step 6.1.4), analyze total intensities within pixels for every spot using ImageJ.
  4. Calculate the labeling occupancy (LO) using the following formula:

LO= (number of counts in the sample / number of counts in the positive control) x 100

* 1. Calculate the average number of fluorescent dyes bound to a single protein molecule (*n̄*dye).
     1. Analyze a histogram of intensities of every spot.

NOTE: The histogram usually has several peaks, and every peak represents a different number of dye molecules bound to a protein. The first, second and third peak in the histogram corresponds to 1, 2 and 3 molecules, respectively.

* + 1. Calculate the average intensity from the histogram. Also calculate the peak intensity of the first peak by applying a Gaussian fitting.
    2. Calculate *n̄*dye by dividing the average intensity with the peak intensity.

# **REPRESENTATIVE RESULTS**

**Figure 4** represents raw image data for different molecular weight fractions of proteins from HeLa cell lysate, as well as the positive and negative control. While both the protein sample and positive control exhibit 100–500 spots per image, the negative control displays none or a few spots, showing that the protocol sufficiently inhibits non-specific binding of dyes to the coverslip surface. Spot intensity histograms for the protein samples and the positive control present multiple peaks, representing stochastic binding of dyes to primary amines in proteins and avidin tetramer structures[22](https://paperpile.com/c/2NQckN/njomU), respectively. All spots showed blinking and stepwise photobleaching with continuous laser excitation, highlighting observation at the single molecule level (**Figure 5A,B**).

The LO is calculated from the ratio of the number of spots in every protein sample to that in the positive control. The LO measured from the HeLa cell lysate sample ranged from 50% for the smaller molecular weight (16 kDa) fraction to 90% for the higher molecular weight (120 kDa) fraction and was 72% for the whole proteome sample without separation (**Figure 6**). Correspondingly, *n̄*dyetended to increase with increasing molecular weight. These increasing tendencies are considered to occur due to higher numbers of lysine residues, leading to increased NHS-ester dye binding. For example, the 23 kDa fraction has a high LO value because of the large numbers of lysine residues in histone proteins contained in this fraction.

# **FIGURE LEGENDS**

**Figure 1: Effect of proteome labeling homogeneity on protein number counting.** The protein count number is highly dependent on how strongly and homogeneously protein molecules are labeled, rather than the number ratio of proteins to dyes. The homogeneity can be scored using a parameter termed labeling occupancy (LO), which defines the probability of labeled protein molecules against total protein molecules. This value provides the efficiency of protein counting (i.e., higher LO yields higher count numbers (left) and vice versa (right)). While a LO value of 100% is ideal, LO of <100% can provide an attenuation factor for estimating absolute protein numbers. This figure has been modified from Leclerc et al.[16](https://paperpile.com/c/2NQckN/YUzCx) Copyright 2018 American Chemical Society.

**Figure 2: Assay workflow.** First, coverslips (**a**) and (**b**) are treated with avidin to achieve a fixed density. Second, fluorescently-labeled sample proteins are biotinylated and attached to the coverslip (**a**) via the avidin-biotin interaction. In parallel, nearly 100% fluorescently-labeled purified biotin is immobilized on the coverslip (**b**). Third, the coverslips are imaged by single-molecule fluorescence microscopy to obtain the number and brightness distribution of fluorescence spots. Finally, the homogeneity parameter,LO, is calculated from the ratio of spot numbers in (**a**) to that in (**b**). This figure has been modified from Leclerc et al.[16](https://paperpile.com/c/2NQckN/YUzCx) Copyright 2018 American Chemical Society.

**Figure 3: Separation of a HeLa cell proteome sample using SDS-PAGE.** Fluorescently labeled and biotinylated cell lysate were migrated on two different gels (20% acrylamide), and proteins visualized using the gel viewer, with a 5 min and 10 min exposure time for gel 1 and gel 2, respectively. The red boxes represent the gel regions that were cut out and extracted. This figure has been modified from Leclerc et al.[16](https://paperpile.com/c/2NQckN/YUzCx) Copyright 2018 American Chemical Society.

**Figure 4: Single molecule images of proteome samples.** Fluorescence spot images (left) and histograms of spot intensities (right) obtained from different molecular weight proteome fractions. The scale bar is 10 μm. Arrows in the positive control indicate the different labeling step of avidin. This figure has been modified from Leclerc et al.[16](https://paperpile.com/c/2NQckN/YUzCx) Copyright 2018 American Chemical Society.

**Figure 5: Photobleaching of fluorescent spots.** (**A**) Time-lapse images of fluorescent spots under continuous laser excitation. (**B**) Time traces of fluorescence intensities at different spots. The traces show either one- or two-step photobleaching, indicating respective numbers of dyes were coupled to the protein in the spot of purified BSA sample. This figure has been modified from Leclerc et al.[16](https://paperpile.com/c/2NQckN/YUzCx) Copyright 2018 American Chemical Society.

**Figure 6: Labeling homogeneity of proteome samples.** LO(blue) and *n̄*dye (dark red) obtained from different molecular weight fractions. Data are expressed as mean ± s.e. 98, 46, 27, 77, 44, 39, 62, 101, 65, 62, 82, 61 and 70 images were analyzed for the whole cell lysate, 16, 23, 55, 120, 19–25, 25–32, 32–42, 42–54, 54–69, 69–97, 97–136 and 136–226 kDa fractions, respectively. This figure has been modified from Leclerc et al.[16](https://paperpile.com/c/2NQckN/YUzCx) Copyright 2018 American Chemical Society.

# **DISCUSSION:**

This paper describes a protocol to quantify the labeling homogeneity of each labeled protein species in cells after separation with SDS-PAGE (step 3). The separation method can be substituted with other methods such as liquid chromatography or capillary electrophoresis, which allow separation and fractionation of cellular proteins with high resolution, while requiring special equipment[23](https://paperpile.com/c/2NQckN/8aaU). The labeling method using NHS-ester in the current protocol can be replaced with other methods using maleimide-ester or antibodies, for example.

The requirement for labeling homogeneity analysis depends on how much the labeling deviates from a simple random process, as assumed in the coupling efficiency analysis. Our recent study[16](https://paperpile.com/c/2NQckN/YUzCx) indicated that the NHS-ester labeling of proteins deviates from a random process depending on the reaction conditions such as pH and detergents. We observed that some conditions induced heterogeneity by cooperative binding of dyes to proteins or the existence of a fraction of non-reactive proteins due to incomplete solubilization or lowered affinity for the dye. By contrast, other conditions reduced heterogeneity by the complete binding of reactive residues within a protein to the dye.

We consider that pH is one of the most influential factors on the labeling homogeneity. High pH can cause neutralization of lysine residues in proteins, leading to a higher reactivity with the NHS-ester dye. In addition, high pH can induce cell lysis and can denature proteins. However, since high pH makes the charge of proteins negative and is not suitable for some experiments such as the isoelectric electrophoresis or 2D electrophoresis, the pH condition needs to be carefully considered depending on the research purpose.

To precisely measure the labeling homogeneity in the assay, it is important to use enough biotinylated protein sample. This is because the assay assumes that proteins bind with saturation to every avidin molecule on the coverslip, and insufficient protein concentration results in underestimation of LO. Our previous study[16](https://paperpile.com/c/2NQckN/YUzCx) has shown that typically more than 10 pg of protein is necessary for saturated binding, and this can be obtained from around 1,000 HeLa cells[24](https://paperpile.com/c/2NQckN/X7vH), even after fractionation. It is recommended to create a dilution series of the protein sample to confirm the saturation of avidin spots. We also note that the avidin density on the coverslip may need to be optimized so as not to cause overlaps between spots in recorded images, while giving sufficient spot numbers for statistical analysis.

This assay assumes that one tetravalent avidin molecule[22](https://paperpile.com/c/2NQckN/njomU) on the coverslip can bind one protein. Indeed, the positive control data in our previous study indicated that one avidin molecule can bind to up to four fluorescently labeled biotins, but the majority (55%) of avidin molecules bind only one or two biotin molecules. Because proteins are larger than biotin, their steric effects should result in fewer proteins bound to one avidin. Our assumption is further supported by the fact that *n̄*dye remains constant when measuring mixed samples of labeled and unlabeled proteins at different ratios[16](https://paperpile.com/c/2NQckN/YUzCx).

Depending on the protein species, unspecific binding of proteins to the coverslip might occur significantly even with a BSA coating. If this is the case, one option may be to measure a control where biotinylated proteins are coated on the coverslip without avidin, instead of fluorescent biotin at step 4.6.3. The effect of the unspecific binding on LO can be mathematically eliminated by subtracting the number of spots of this control from that of the protein sample.

This protocol will be invaluable for proteome-wide single-molecule protein counting analyses. The single molecule sensitivity will allow analysis of protein amounts for every protein species irrespective of their abundance in the cell[25](https://paperpile.com/c/2NQckN/YicEu). This could be realized by proteome separation followed by quantification of the number of fluorescence spots coupled with proteins. Further, the high sensitivity will also allow single cell analysis, enabling researchers to examine heterogeneity across cell populations and to do clustering of cellular states[25,26](https://paperpile.com/c/2NQckN/qMhyg+YicEu).

# **ACKNOWLEDGEMENTS**

The authors thank Masae Ohno and Kazuya Nishimura for experimental assistance and advice. This work was supported by PRESTO (JPMJPR15F7), Japan Science and Technology Agency, Grants-in-aid for Young Scientists (A) (24687022), Challenging Exploratory Research (26650055), and Scientific Research on Innovative Areas (23115005), Japan Society for the Promotion of Science, and by grants from the Takeda Science Foundation and the Mochida Memorial Foundation for Medical and Pharmaceutical Research. S.L. acknowledges support from the RIKEN International Program Associate (IPA) program.

# **DISC**LO**SURE**

The authors declare the following competing financial interest(s): RIKEN has filed a patent application on these results with S.L. and Y.T. named as co-inventors.

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