Journal of Visualized Experiments

Proteome-wide quantification of labeling homogeneity at the single molecule level

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59199R1
Full Title:	Proteome-wide quantification of labeling homogeneity at the single molecule level
Keywords:	Single molecule imaging, proteomics, fluorescence labeling, gel electrophoresis, labeling homogeneity, cell lysate
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Additional Information:	
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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]. 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]. 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].

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]. 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] 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]. 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], and the average number of fluorescent dyes bound to a single protein molecule ($\bar{n}_{dye}$), 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:

1. Cell preparation

1.1. Cultivate HeLa cells in a 10 cm dish at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

1.2. Collect exponentially growing cells once they have reached 70% confluency, following ATCC instructions[17].

1.2.1. Rinse cells with 5 mL of 1x phosphate buffered saline (PBS) (pH 7.4). Remove PBS.

1.2.2. Add 1 mL of 0.1% trypsin-ethylenediaminetetraacetic acid (EDTA). Incubate 5 min at 37 °C.

1.2.3. Monitor the progression of cell dissociation by microscopy.

1.2.4. Once cells become round and are detached from the dish, add 4 mL of growth medium, and break cell agglomeration by pipetting.

1.2.5. Count the number of cells using a cell counter.

1.2.6. 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).

1.2.7. Repeat step 1.2.6. Resuspend cells in 1x PBS (pH 7.4) to a final concentration of 10^6 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.

2. Cell lysis and fluorescent labeling

2.1. 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.

2.2. 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).

2.3. 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.

2.4. 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.

2.5. 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.

2.6. 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.

2.7. 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.

2.8. 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.

3. Proteome separation and recovery

3.1. 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.

3.2. 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.

3.3. Image the gel to identify the location of the protein bands (**Figure 3**).

3.4. 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**.

3.5. 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.

4. Microscope coverslip preparation

4.1. 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.

4.2. 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.

4.3. 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.

4.4. Prepare coverslips for the protein sample.

4.4.1. Dilute the protein sample (from 3.5) 100 times in 5 mM HEPES-NaOH pH 7.2.

4.4.2. Place 100 µL of the diluted sample on the surface at the center of the avidin-coated coverslip (from step 4.3).

4.4.3. Incubate in the dark for 15 min at room temperature.

4.5. Prepare the positive control.

4.5.1. 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).

4.5.2. 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.

4.6. Prepare the negative control.

4.6.1. Spin-coat the plasma cleaned coverslip with 200 µL of 5 mM HEPES-NaOH and 2 mg/mL BSA (without avidin).

4.6.2. Incubate for 15 min at room temperature to dry.

4.6.3. Add 100 µL of 10 ng/mL purified fluorescent biotin in 5 mM HEPES-NaOH pH 7.2.

4.6.4. 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.

4.7. 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.

4.8. Expose air plasma to the same number of coverslips as in step 4.2.

4.9. Place the cleaned coverslip on the sample-bound coverslip from step 4.7 to avoid drying.

5. Observation with single molecule fluorescence microscopy

5.1. 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]. 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.

5.2. Set the coverslip on the microscope and find the focus.

5.3. 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.

6. Image analysis and extraction of information

6.1. If the image has unevenness due to the laser illumination pattern, carry out image processing to correct it[18].

6.1.1. 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.

6.1.2. Acquire an offset image by measuring with no laser excitation using the same camera setting.

6.1.3. Subtract each pixel value in every sample image and the reference image with the value of the offset image.

6.1.4. Divide each pixel value in the subtracted sample images by the value in the subtracted reference image.

6.2. Collect properly acquired images and remove the image background.

6.2.1. Remove images containing large fluorescent aggregates manually.

6.2.2. Remove the image background by applying the rolling-ball algorithm[19] with a ball radius of 50 pixels using ImageJ[20].

6.2.3. Sharpen images by subtracting blurred images using ImageJ (sharpen image function).

6.3. Find and analyze single molecule spots in images.

6.3.1. Identify single molecule spots with the Yen threshold algorithm[21] using ImageJ.

6.3.2. 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).

6.3.3. 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.

6.4. 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

6.5. Calculate the average number of fluorescent dyes bound to a single protein molecule ($\bar{n}_{dye}$).

6.5.1. 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.

6.5.2. Calculate the average intensity from the histogram. Also calculate the peak intensity of the first peak by applying a Gaussian fitting.

6.5.3. Calculate $\bar{n}_{dye}$ 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], 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, $\bar{n}_{dye}$ tended 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] 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] 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] 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] 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] Copyright 2018 American Chemical Society.

Figure 6: Labeling homogeneity of proteome samples. LO (blue) and $\bar{n}_{\mathrm{dye}}$ (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] 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]. 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] 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] 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], 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] 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 $\bar{n}_{\mathrm{dye}}$ remains constant when measuring mixed samples of labeled and unlabeled proteins at different ratios[16].

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]. 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].

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.

DISCLO**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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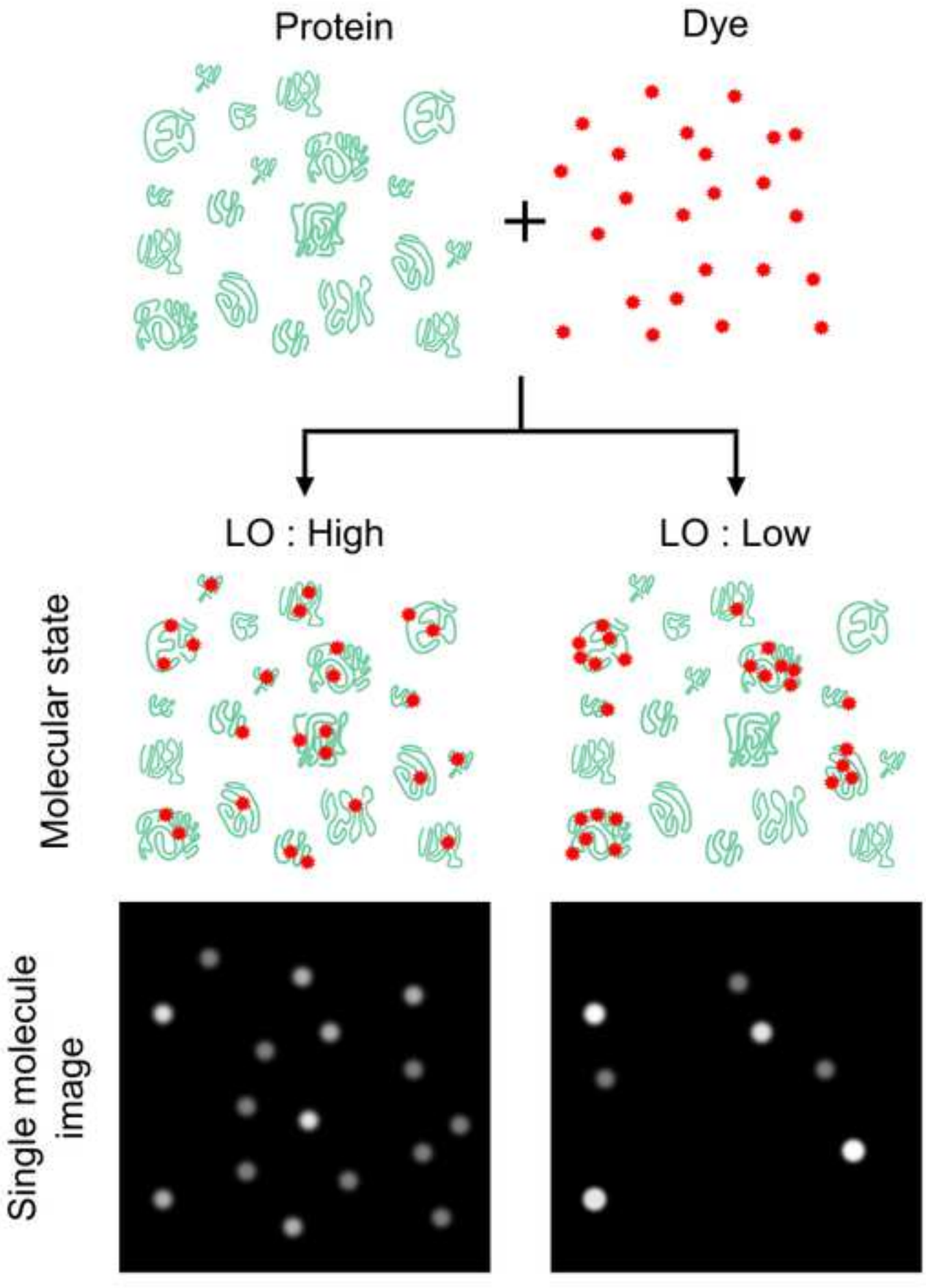

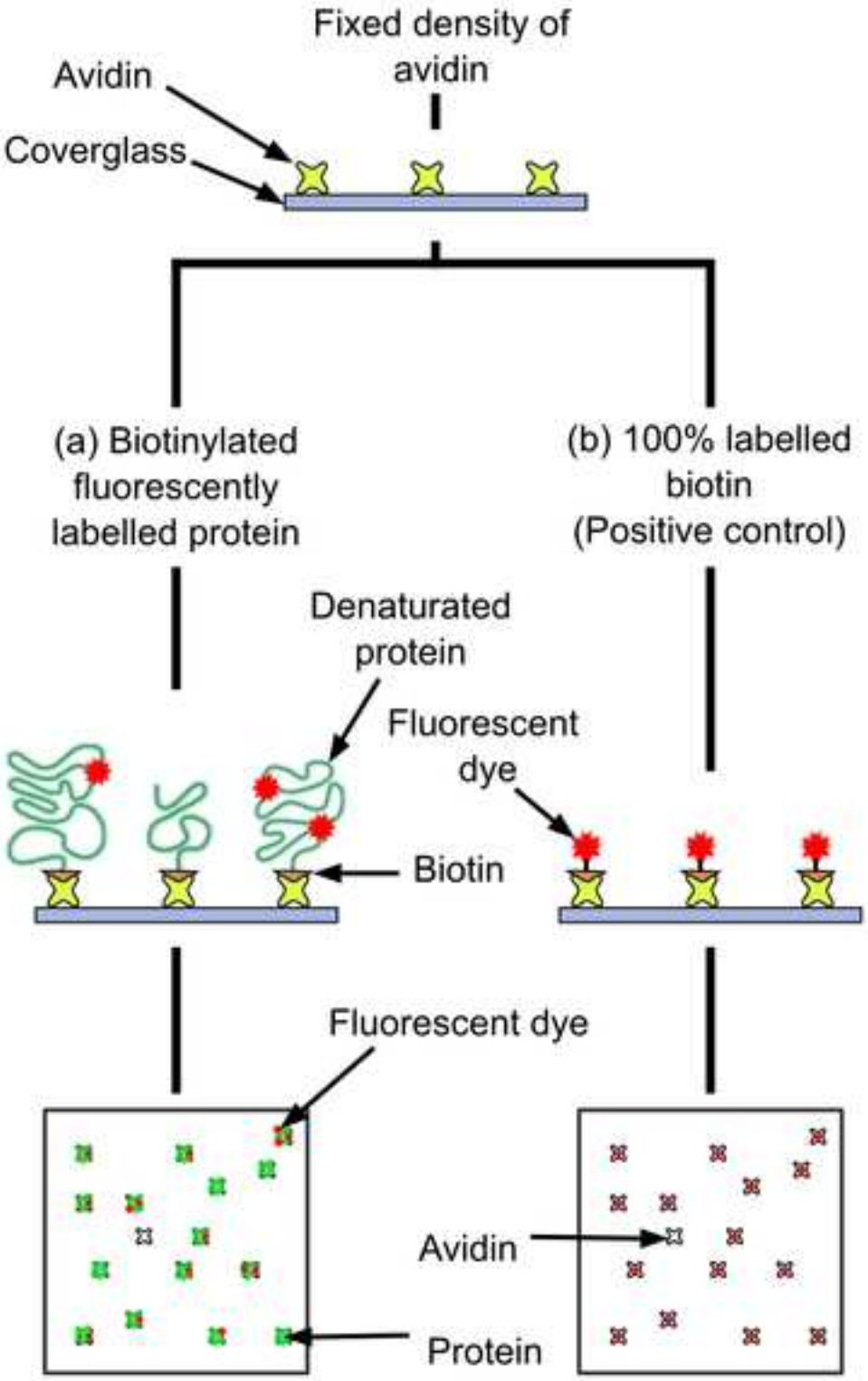

$$LO = \frac{\text{\# of count in (a) x 100}}{\text{\# of count in (b)}}$$

Figure3

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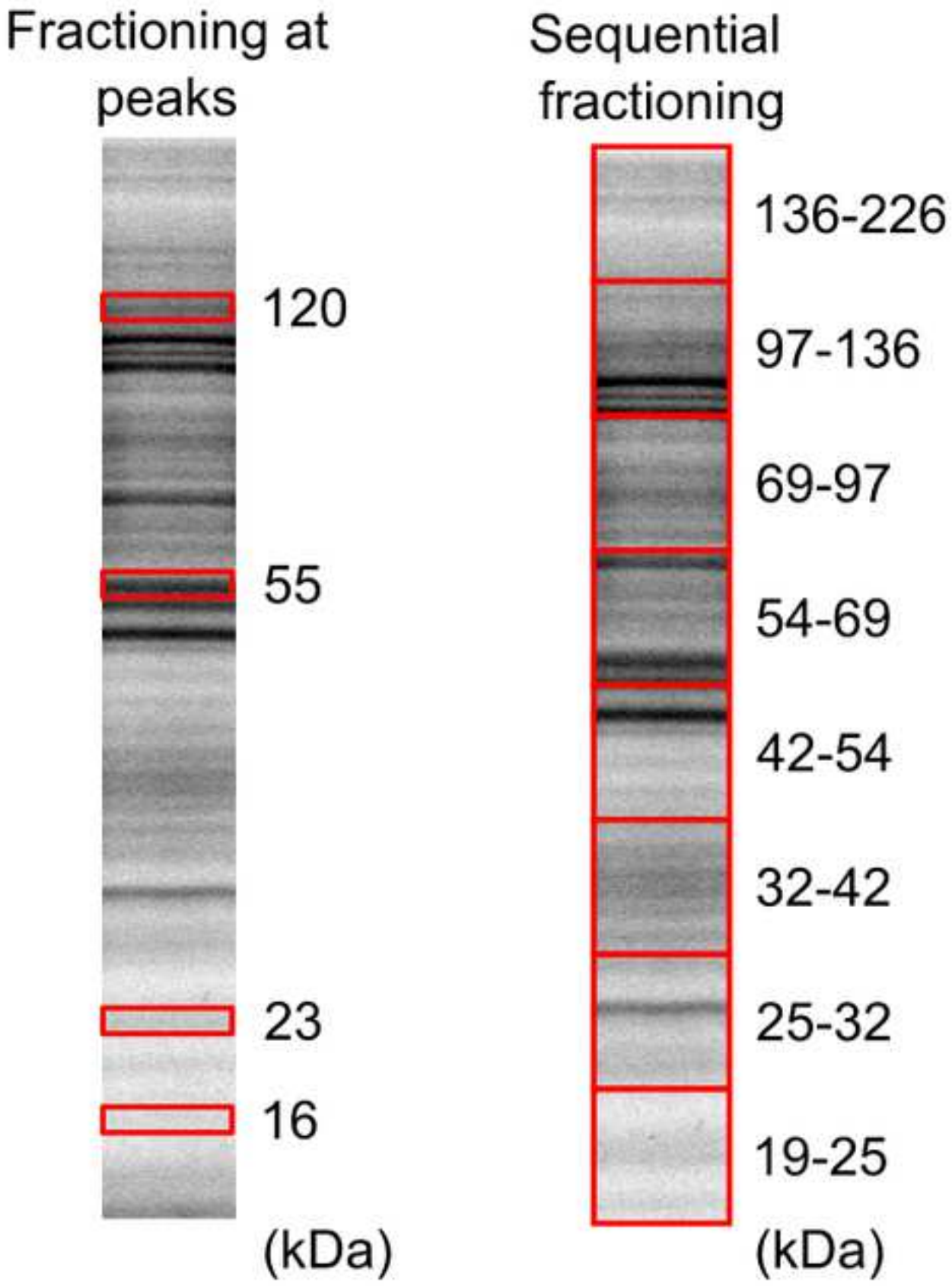

Figure4

Click here to access/download;Figure;fig4.tif

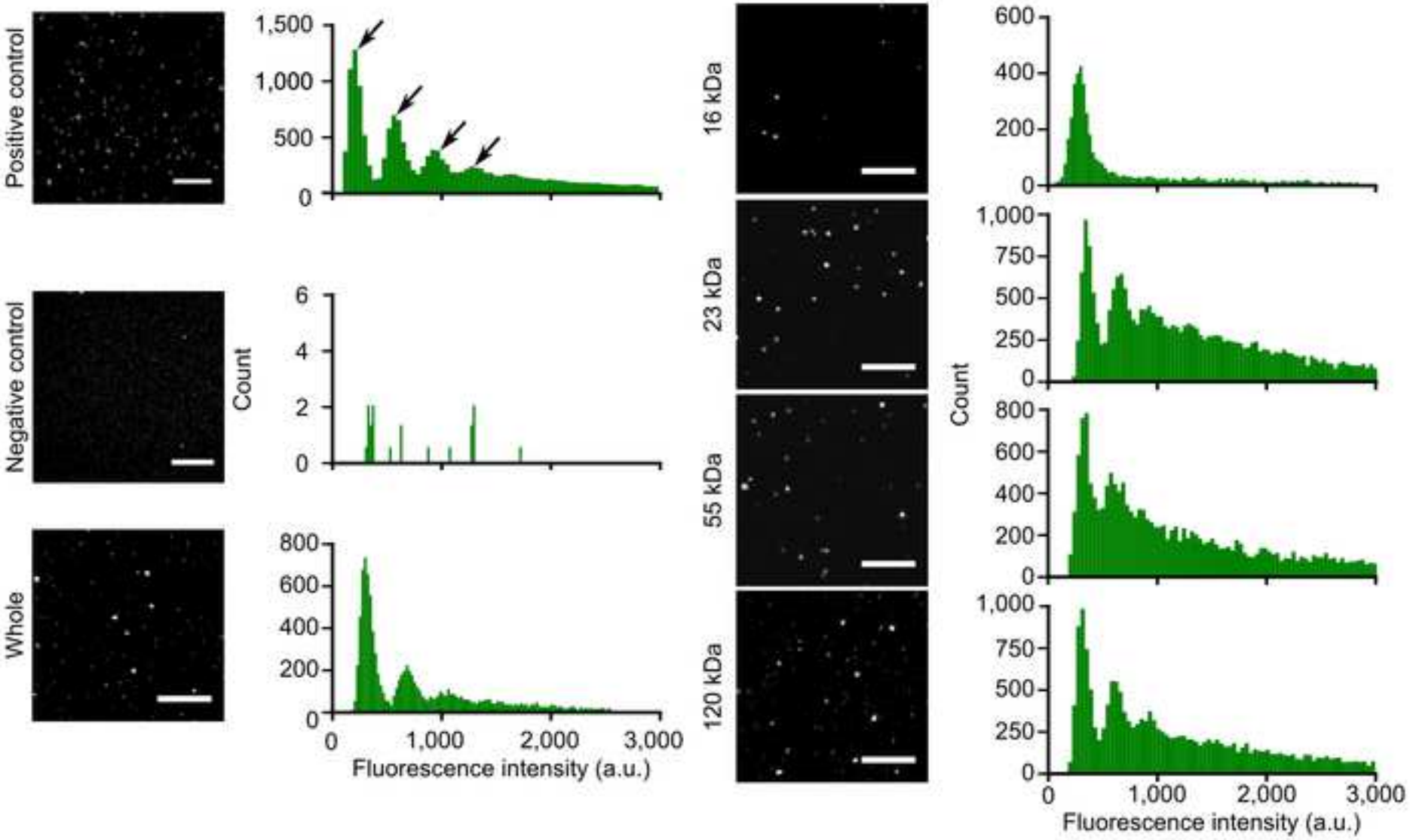

Figure5

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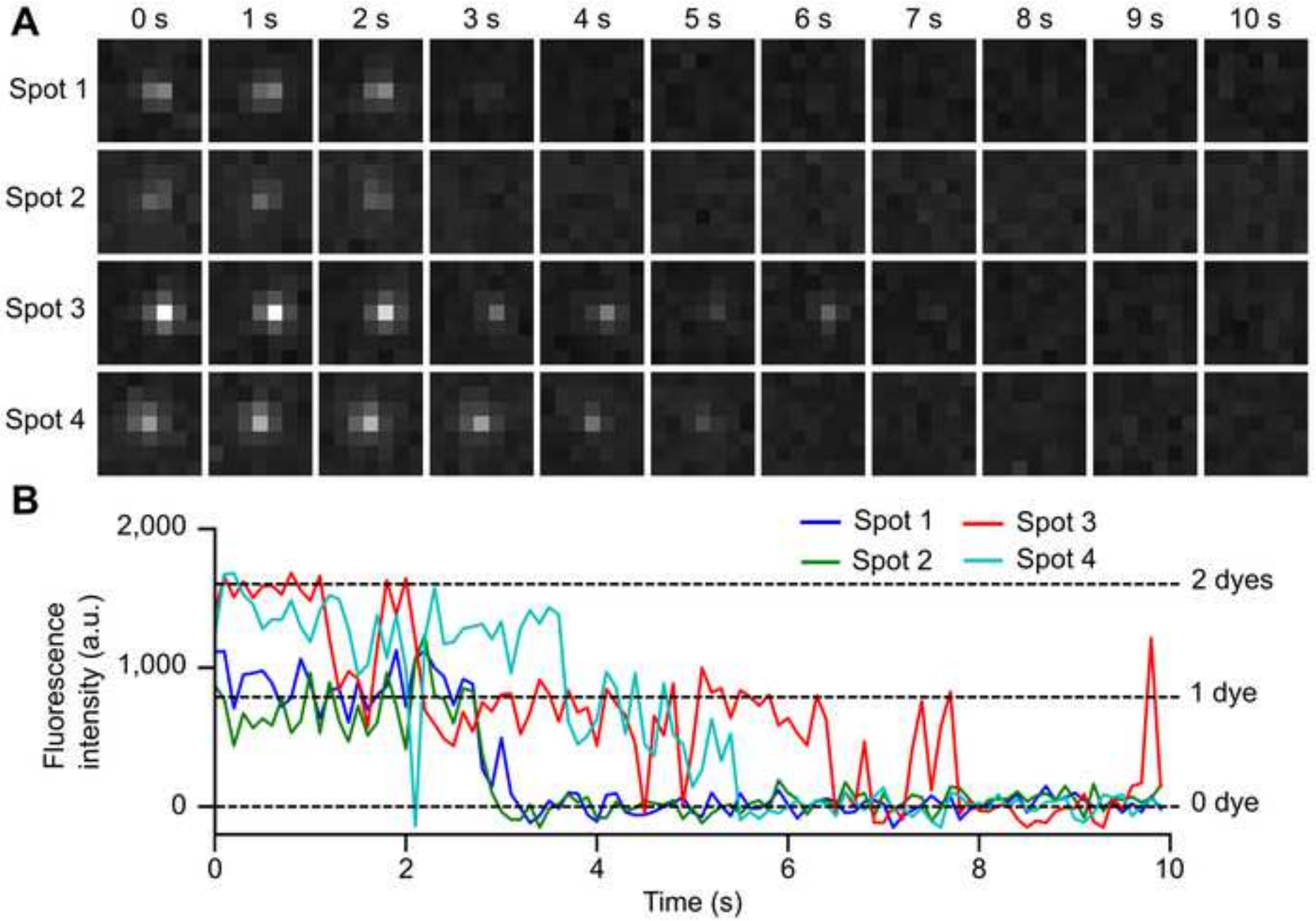

Figure6

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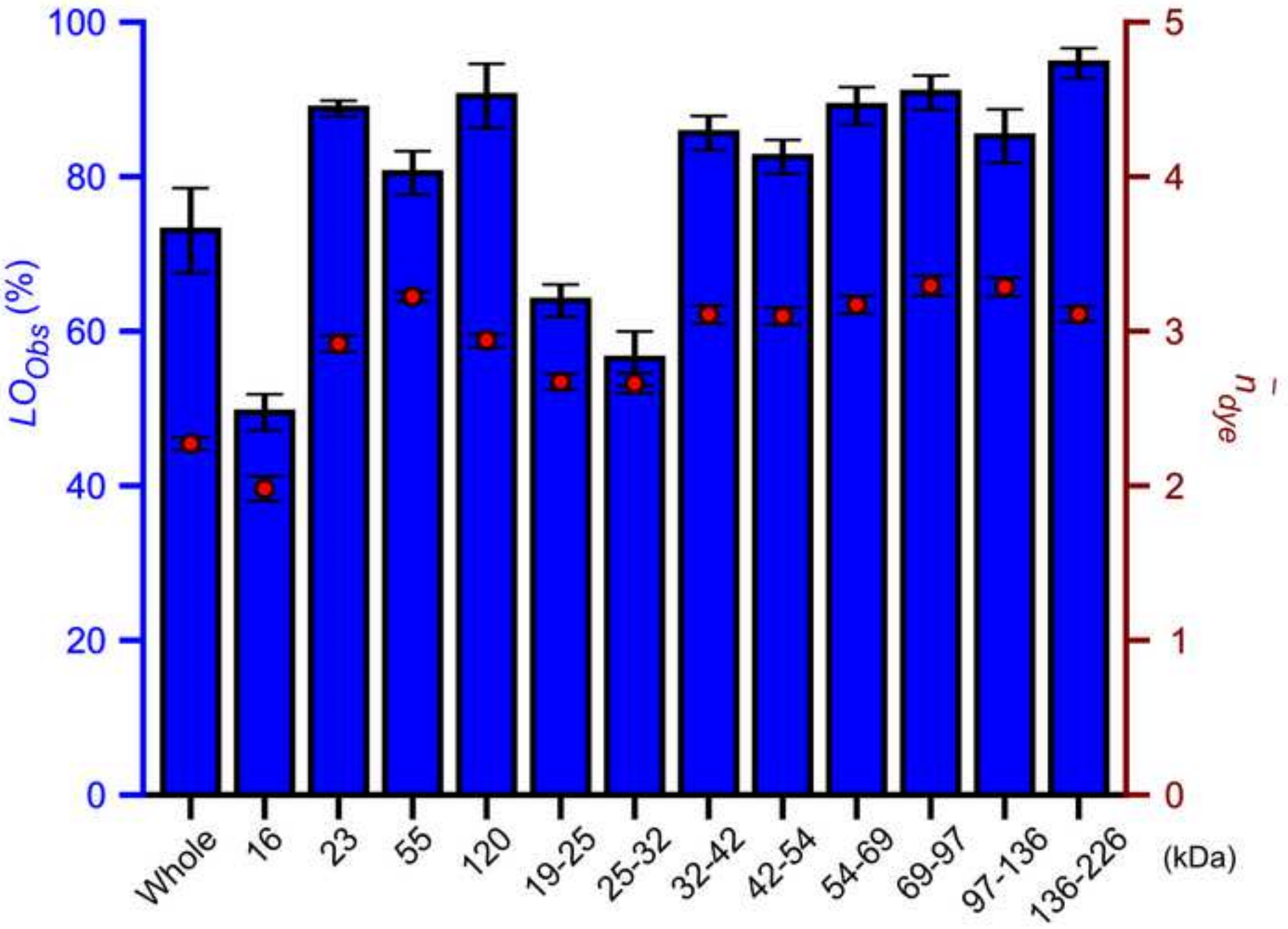

Figure1

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Fig1.svg

Figure2


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Figure3

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Figure4

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fig4.svg

Figure5

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fig5.svg

Figure6


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Video or Animated Figure
fig6.svg

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
22x22x0.15 mm coverslip	VWR	470019-004	
488 nm Argon laser	Coherent	Innova 70C	
488 nm dichroic mirror	Semrock	FF495-Di03	
488 nm emission filter	Semrock	FF02-520/28	
560 nm dichroic filter	Semrock	Di02-R561	
560 nm emission filter	Semrock	FF02-617/73	
560 nm fiber laser	MBP Communications	F-04306-2	
60x oil immersion lens	Olympus	PLAPON 60x	
Avidin	Nacalai-tesque	03553-64	
Biotin-PEG-amine	Thermo Scientific	21346	
Biotinylated Alexa Fluor 488	Nanocs		
Borate	Nacalai-tesque		
BSA	Sigma-Aldrich	A9547	
CHAPS	Dojindo	C008-10	
Cy3 NHS-ester dye	GE Healthcare	PA13101	
Dialyzer - D-tube, 6-8 kDa	Merck Millipore	71507-M	
DMEM	Sigma-Aldrich		
DTT	Nacalai-tesque	14112-94	
EDC	Nacalai-tesque		
EMCCD camera	Andor	IiXon 897	
Epi fluorescence microscope	Olympus	IX81	
Gel viewer	GE Healthcare	4000	
Penicillin, streptomycine and Amphoterecin mix	Gibco		
Plasma cleaner	Diener Electronic		
SDS	Wako	NC0792960	
Size purification column 10K	Merck Millipore	UFC5010	
Tween 20	Sigma-Aldrich	P9416	

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Author(s): Simon Lederc, Youri Arntz, Yuichi Taniguchi

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CORRESPONDING AUTHOR:

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Department:	Center for Biosystems Dynamics Research
Institution:	RIKEN
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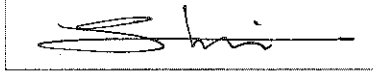
Signature:	
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Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for the suggestion. The manuscript has now been checked using proofreading services, so we believe that there are no spelling/grammar issues.

2. Please revise lines 209-226 to avoid previously published text.

To address this issue, we have revised the discussion section, so as not to include the same sentences as in the previous publication.

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We have uploaded the copyright permission obtained via the Copyright Clearance Center. We have also revised the figure legends to include the phrase “This figure has been modified from”, followed by the citation information.

4. Please provide an email address for each author.

The email address for each author is:
- leclerc@uthscsa.edu (Simon Leclerc)
- youri.arntz@unistra.fr (Youri Arntz)
- taniguchi@riken.jp (Yuichi Taniguchi)

5. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

We have added a Summary section:
“Here, we present a protocol to assess the labeling homogeneity for each protein species in a complex protein sample at the single molecule level.”

6. Please define all abbreviations (DTT, CHAPS, EDC, etc.) before use.

We have added definitions for the abbreviations in the main text, including FBS, PBS, EDTA, SDS, DTT, CHAPS, HEPES, PEG and EDC.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Thank you for the suggestion. We have revised the protocol to include only the imperative tense. Text that cannot be written in the imperative tense was moved to a “Note” or the discussion section. We have also described all safety procedures in the text, including laser safety.

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We revised descriptions for cell collection (step 1.2) by providing a reference and details with multiple steps. We also provided further details for image analysis (step 6).

9. 1.2: Please specify centrifugation parameters (force in x g and time) as well as reaction conditions of trypsinization.

We added centrifugation parameters in step 1.2.6. We also described the reaction conditions of trypsinization in step 1.2.2.

10. 2.4: Please describe how to homogenize the solution.

We added the description “by slowly pipetting to avoid bubbles” to step 2.4. We also added the same description to steps 2.5, 2.6 and 2.7.

11. 3.1: Please provide the composition of SDS sample buffer.

We have added the composition of SDS sample buffer (200 mM Tris-HCl pH 6.8, 4% SDS, 4% glycerol and 0.4% bromophenol blue) to step 3.1.

12. 3.4: Please specify specific protein bands selected in this protocol.

We have noted the band information in step 3.4.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have added single-line spaces in the revised manuscript.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted essential steps of the protocol in yellow.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have adhered to the above points regarding the highlighting.

17. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We have discussed critical steps in the protocol in the Discussion section. Particularly, we consider the avidin and biotinylated protein glass coating step to be critical, so we created a new paragraph to discuss this (the paragraph starting from “To precisely measure”).

18. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

We have sorted the items in alphabetical order in the Table of Equipment and Materials.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript is basically a short version of a recently published paper by the authors in bioconjugation chemistry, with all figures taken from this publication (with reference and copyright statement).
The authors describe a method that allows for the analyses of the efficiency and homogeneity of fluorescent labelling of proteins samples and apply the technique to HELA lysates. It describes an initial step of Cy3 labelling of the protein sample (the labeling whose efficiency will be analyzed). Next they label the protein sample with Biotin. This will allow the protein sample to bind to a avidin labeled microscope slide later in the protocol. Once labelled, the proteins sample is separated using SDS-PAGE, electro-eluted, and spotted on a microscope slide that is coated with avidin. Next, sample can be analyses by fluorescent microscope. Since this procedure produces individual spots, that according to the authors represent single molecules, for each spot the intensity can be determines, which should be step-wise increase depending on how many fluorophores have been conjugated onto that individual molecule. Besides the ability to determine the average number of fluorophores attached, the authors can also determine how many of the molecules have been labelled by comparing the number of fluorescent spots with a positive control that is 100% labeled. Here the assumption is that the number of avidin spots on each slide is identical, so no fluorescent spots are occupied by Biotin-labeled protein that lacks fluorophore label.

Overall, the technique is interesting and valuable, as I think it is important for researchers to know what fraction of their proteins is labeled. That said, some sections were thin on their description, which might be addressed in the video production and maybe a good reason to warrant a video protocol as I cannot readily envision from my experimental background what or how it takes place (e.g.line 130 up air plasma and microscope slide treatment).

Major Concerns:
-The third lane in figure 2 is identical to others, but shifted. One band visible is all three lanes is labeled 55 kDa in lane 2 and in lane three it is at the top of a molecular range section that spans from 54-69, suggesting it is 69 kDa. This is impossible and something is wrongly assigned.

We thank the reviewer for this important point. We agree that the 55 kDa band was wrongly assigned. We have corrected the indication of the 55 kDa band in the figure.

-Some discussion of limitations or some key potential pitfalls would be valuable.
-Technically I was not completely sure on what limits the spots on the slide, is there limited avidin, or is the protein concentration so low that there is only limited binding? I saw the supplement of the original paper has some titration curve, where depending on the protein concentration the binding plateau's.

Also, doesn't avidin bind 4 biotin molecules, leaving the potential for four molecules at a spot instead of one? As the protocol is published and the current version has limited technical details, I did not dive into a full critical analyses here, but more general I think the manuscript would benefit from discussion of some potential pitfalls or points of caution. Comparing a variety of very different conditions with a very different independent positive control can be tricky and I was wondering how crucial are accurate protein concentration measurements etc. There seems to be potential for misleading result sin certain steps are not carefully executed at the right concentration. Some discussion or clarification here would be valuable.

Thank you for the useful suggestions. Although some of these questions were discussed in the original paper, we agree that it is important to cover them in this manuscript. As the referee may expect, the spot number is limited by the number of avidin molecules attached to the slide, because subsequent binding of proteins to avidin is saturated. Therefore, in our assay, it is important to use a sufficient quantity of biotinylated protein sample, and too little sample results in underestimation of *LO*. The avidin density on the slide has been optimized to provide sufficient spot numbers for statistical analysis with no overlaps between spots in images.

As for the avidin binding site issue, as the referee argued, this assay assumes that one tetravalent avidin molecule on the coverslip can bind one protein. Indeed, the positive control data in our previous study (Leclerc et al., 2018) indicated that one avidin molecule can bind to up to four fluorescently labeled biotin molecules, 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 molecule. Our assumption is further supported by the observation in the previous study that $\underline{n}_{\mathrm{dye}}$ remains constant when measuring mixed samples of labeled and unlabeled proteins at different ratios. We added these arguments to the Discussion section (the paragraphs starting with “To precisely measure” and “This assay assumes”).

Minor Concerns:
-How do authors know that positive control is truly 100% labelled?
-Is the biotin labelling 100%? Probably does that not matter as long as there is no bias in the labelling?

We referred to this as “100% labeled” because the product was created through chemical purifications following biotin conjugation reactions. In fact, the company assures at least 95% purity (http://www.nanocs.com/PEG/BPEG.htm). To avoid confusion, we have changed “100%” to “nearly 100%”.

-The authors in the original manuscript show a nice overview cartoon in figure 2 that would be a helpful overall picture of the protocol.

We agree with the reviewer. We have included the overview cartoon as a new Figure 2.

-Especially for a methods story care should be taken to clear and accurate description. Eg. Line 81. It is ambiguous if samples should be stored as pellet or as PBS resuspended sample. I assume pellet, but better to be clearly stated. Line 114 incubate at RT?

We agree that some descriptions were ambiguous. In Line 81, PBS resuspended sample was aliquoted. In Line 114, the sample was kept at room temperature. We have revised these descriptions.

-There seems to be only limited discussion on alternative ways people have addressed this, what other approaches have people used?

Thank you for the suggestion. In our protocol, the protein separation step with SDS-PAGE can be substituted with other methods such as liquid chromatography or capillary electrophoresis. In addition,

the labeling method using NHS-ester can be replaced with other methods using maleimide-ester or antibodies. We have added these alternatives to the discussion section (the paragraph starting with “This paper describes”).

-Many awkward or unusual English sentence constructions are used, need to be editing, e.g. line 111 The protocol can terminate here.

This version of the manuscript has been proofread by a commercial English editing service (Life Science Editors, https://lifescienceeditors.com/).

-Title is hard to read.

We changed the title to ”Proteome-wide quantification of labeling homogeneity at the single molecule level”.

Original: Proteome-wide labeling homogeneity of a complex protein sample at the single molecule level

-Line 47 is CB a fluorescent dye?

Coomassie blue (CB) is indeed more often used as a visible dye, but it can also be used as a fluorescent dye. We added an additional reference demonstrating this (Butt and Coorssen, 2013).

-section 6 is important for the protocol as it is the key analyses of the data, but rather limited in its description.

We have added descriptions for the image analysis in section 6. In particular, we have added descriptions for laser pattern correction (step 6.1) and derivation of $\overline{n}_{\mathrm{dye}}$ (step 6.5), and image analysis parameters for background subtraction (step 6.2) and spot filtering (step 6.3).

Reviewer #2:
Manuscript Summary:
The authors describe a method to determine labeling homogeneity of nonspecific labels across proteins in biological samples. Such labels are used for detection in SD gel electrophoresis based assays, but labeling efficiency has typically been determined at the bulk level. This method enables labeling heterogeneity to be determined. This work should be of interest to those performing labeling studies of proteins.

Major Concerns:
None

Minor Concerns:
-The authors state on Line 38 that "Proteome analysis, which quantifies the entire set of protein molecules expressed in the cell, is a demanded approach in current biological and medicinal studies." In fact, proteomic analyses never quantify the entire set of proteins despite efforts to do so. We only ever quantify a fraction of the ~20000 expressed proteins. This should be corrected.

We agree with the reviewer. We have changed the expression “which quantifies the entire set” to “which aims to quantify the entire set”.

-Mass spectroscopy on Line 40 should be changed to mass spectrometry.

Thank you for the suggestion. The word has been corrected.

-The rest of the manuscript is in need of some copy editing.

This version of the manuscript was copy-edited using a professional proofreading service.

Title: Extending Single Molecule Imaging to Proteome Analysis by Quantitation of Fluorescent Labeling Homogeneity in Complex Protein Samples

Author: Simon Leclerc, Youri Arntz, Yuichi Taniguchi

Publication: Bioconjugate Chemistry

Publisher: American Chemical Society

Date: Aug 1, 2018

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