Journal of Visualized Experiments

Fluorescent Silver Staining of Proteins in Polyacrylamide Gels

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58669R1
Full Title:	Fluorescent Silver Staining of Proteins in Polyacrylamide Gels
Keywords:	Silver staining; Fluorescent gel staining; Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE); Aggregation induced emission (AIE); Silver ion sensor; Fluorogenic detection; Proteomics
Corresponding Author:	Sijie Chen SWEDEN
Corresponding Author's Institution:	
Corresponding Author E-Mail:	sijie.chen@ki.se
Order of Authors:	Alex. Y.H. Wong
	Sheng Xie
	Ben Zhong Tang
	Sijie Chen
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Hong Kong S.A.R

TITLE:

Fluorescent Silver Staining of Proteins in Polyacrylamide Gels

AUTHORS AND AFFILIATIONS:

Alex. Y. H. Wong[1], Sheng Xie[1], Ben Zhong Tang[2], Sijie Chen[1]

[1]Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet, Hong Kong

[2]Department of Chemistry, Hong Kong Branch of Chinese National Engineering Research Center for Tissue Restoration and Reconstruction, Institute of Molecular Functional Materials, State Key Laboratory of Neuroscience, Division of Biomedical Engineering, and Division of Life Science, The Hong Kong University of Science and Technology, Kowloon, Hong Kong

Corresponding Author:

Sijie Chen (sijie.chen@ki.se)

Tel: +852 2624 3811

E-mail Addresses of the Co-authors:

Alex Y. H. Wong (alex.wong@ki.se)

Sheng Xie (sheng.xie@ki.se)

Ben Zhong Tang (tangbenz@ust.hk)

KEYWORDS:

Silver staining, fluorescent gel staining, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), aggregation-induced emission (AIE), silver ion sensor, fluorogenic detection, proteomics

SUMMARY:

Here, we describe a detailed protocol outlining a new fluorescent staining technique for total protein detection in polyacrylamide gels. The protocol utilizes a silver ion-specific fluorescence turn-on probe, which detects Ag^+-protein complexes, and eliminates certain limitations of traditional chromogenic silver stains.

ABSTRACT:

Silver staining is a colorimetric technique widely used to visualize protein bands in polyacrylamide gels following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The classic silver stains have certain drawbacks, such as high background staining, poor protein recovery, low reproducibility, a narrow linear dynamic range for quantification, and limited compatibility with mass spectrometry (MS). Now, with the use of a fluorogenic Ag^+ probe, **TPE-4TA**, we developed a fluorescent silver staining method for the total protein visualization in polyacrylamide gels. This new stain avoids the troublesome silver reduction step in traditional silver stains. Moreover, the fluorescent silver stain demonstrates good reproducibility, sensitivity, and linear quantification in protein detection, making it a useful and practical protein gel stain.

INTRODUCTION:

Many staining methods have been used to visualize proteins following gel electrophoresis, for example using colorimetric dyes such as Coomassie Brilliant Blue, silver stain, fluorescence, or radioactive labeling[1,2,3,4]. Silver staining is considered to be one of the most sensitive techniques for protein detection requiring simple and cheap reagents. It can be categorized into two major families: the ammoniacal silver stain and the silver nitrate stain[5,6]. In the alkaline ammoniacal silver method, the silver-diamine complex is produced with ammonia and sodium hydroxide and reduced to metallic silver during development using an acidic formaldehyde solution. The stain accommodates efficiently for basic proteins but shows a compromised performance for acidic and neutral proteins and is, furthermore, limited to classical glycine and taurine electrophoretic systems. In comparison, the silver nitrate stains exploit the high bio-affinity of silver ions to protein, primarily the sulfhydryl and carboxyl groups from the side chains, and tend to stain acidic proteins more efficiently[7]. After silver ion binding, a developing solution (typically made of a metal carbonate solution containing formaldehyde and sodium thiosulphate) is applied to reduce silver ions to metallic silver grains, which build up a brown-to-dark color to visualize the protein bands.

Although silver staining has been well known for its versatility and high sensitivity since its development in the 1970s[8], the method is frequently regarded as tricky. Silver-staining methods have time-restricted steps and show low reproducibility. Since the color of silver stain is usually not uniform and dependent on the reduction step, which is hard to control, the silver stain is not a quantitative method and, thus, not recommended for gel comparison study and protein quantification[9]. Methods optimized in sensitivity may utilize aldehydes which can also provide a more uniform staining[10]. However, this is at the expense of further downstream analysis due to the crosslinking of proteins by aldehydes. Fast protocols mostly combine or shorten steps to reduce time, compromising the reproducibility and uniformity of the stain[5]. As a result, there are numerous silver staining variants within protein gel staining, each optimized to suit certain requirements; for example, simplicity, sensitivity, or peptide recovery rate for downstream analysis. These attributes may also have an impact on each other, and satisfying all requirements in one protocol can be difficult.

In this work, we introduce a new fluorescent silver staining method for protein detection in polyacrylamide gel. In this method, we use a fluorogenic probe for silver ions, **TPE-4TA** (**Figure 1**), to visualize the silver-impregnated proteins[11]. **TPE-4TA** is designed by the aggregation-induced emission (AIE) principle. It is non-emissive when dissolved in aqueous solution, but is highly emissive in the presence of silver ions. By replacing the chromogenic development in traditional silver stains with a fluorogenic developing step, the fluorescent silver method enables the robust staining of total proteins with a reduced background.

Furthermore, the fluorescent silver stain showed a good dynamic linear range for protein quantification, which is comparable with the widely-used SYPRO Ruby stain and not achievable with traditional silver stains. The gel can be imaged on commonly used gel documentation systems with an ultraviolet lamp (excitation wavelength: 302/365-nm channel; emission: ~490 - 530 nm) at many biological labs.

PROTOCOL:

1. Preparation of the Gel

Note: The demonstration follows a standard protocol to prepare the gel for staining shortly after SDS-PAGE[12]. In brief, the following steps describe the preparation of the samples and gel electrophoresis.

1.1. Perform SDS-PAGE with 4% - 12% Bis-Tris protein gels (1 mm, 15-well) using a mini gel tank filled with 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer.

1.2. Dilute the samples with a mixture of distilled water, lithium dodecyl sulfate (LDS) buffer, and a sample-reducing agent.

1.3. Load the first lane with double the amount of stock (10 µL), followed by the normal stock amount (5 µL) and a series of twofold dilutions of the stock thereafter (13 dilutions, from 2x to 8192x).

1.4. Run the gel at a constant voltage of 200 V for 30 min.

2. Fixation of the Gel

2.1. After electrophoresis, submerge the gels in a 100-mL solution of 40% ethanol/10% acetic acid on an orbital shaker at 50 rpm at room temperature 2x (each for 30 min), or overnight at 4 °C.

2.2. Wash the gels 3 x 10 min each in ultrapure water in a clean container. The washing step is critical. If the acid from the fixing solution is not removed properly in the fluorogenic developing step, the excessive **TPE-4TA** will be activated by the acid and lead to strong background fluorescence in the gel.

3. Preparation of the $AgNO_3$ Solution and Silver Impregnation of the Gel

3.1. To make the $AgNO_3$ solution (0.0001%) for the impregnation, first, dissolve 0.01 g of $AgNO_3$ in 10 mL of ultrapure water to prepare a 0.1% $AgNO_3$ stock solution. Next, add 100 µL of the 0.1% $AgNO_3$ stock solution into 100 mL of ultrapure water to make the working solution.

Note: The $AgNO_3$ solution should be stored in the dark before use.

3.2. Impregnate the gel with 100 mL of silver working solution for 1 h on an orbital shaker at 50 rpm in a sealed glass container. It is critical to perform the silver impregnation under a fume hood, protected from light with aluminum foil.

3.3. Wash the gel with ultrapure water (about 100 mL) in a clean container for 2 x 60 s.

4. Fluorogenic Development of the Gel

4.1. To prepare the dye stock solution (0.1 mM), add 3.0 mg of the **TPE-4TA** dye in 50 mL of ultrapure water. Sonicate the solution for 3 min and add a few drops of a basic solution (for example, 1 mM NaOH) to help dissolve the dye.

4.2. Check the fluorescence of the solution under a 365-nm UV lamp to ensure that the dye molecule is fully dissolved. Only weak or nonemissive solutions indicate full dissolution.

Note: The dye **TPE-4TA** was synthesized following the protocol recently reported by Xie *et al.*[10] The **TPE-4TA** solution is very stable and can be kept in the dark for months.

4.3. To prepare 100 mL of the fluorogenic developing solution (10 μM), add 10 mL of the **TPE-4TA** stock solution into 90 mL of ultrapure water. Check the pH of the solution using a pH meter and tune it to 7 - 9 using a sodium hydroxide solution (1 μM).

4.4. Transfer the gel to a clean and sealable container with 100 mL of the fluorogenic developing solution. Make sure the gel is totally immersed in the solution. Seal the container. Cover it from light and shake it overnight on an orbital shaker at 50 rpm at room temperature. Alternatively, the incubation step can also be shortened to about ~2 h by preheating the AIE developing solution to 80 °C for staining and then leaving it at room temperature.

5. Destaining and Imaging

5.1. Transfer the gel to a clean container and destain it in 100 mL of 10% ethanol for 30 min.

Note: Water alone can be used to wash the gel. However, it is more time-efficient to use a 10% ethanol solution for the destaining. This will help to reduce the destaining process from hours to 30 min.

5.2. Rinse the gel in ultrapure water for 5 min.

5.3. Image the gel at the 365-nm channel or 302-nm channel by a gel documentation machine.

REPRESENTATIVE RESULTS:

The protein bands stained by the fluorescent silver stain exhibit an intense green fluorescence under a 365-nm UV lamp. All 14 protein bands (10 - 200 kDa), from top to bottom, were clearly visible, correlating well with the 14 red-colored ones stained by the SYPRO Ruby dye (**Figure 2**)[10].

Regarding quantitative protein detection, the gels were imaged by a gel imaging system using automated procedures, and the images were analyzed and compared using the commercial software. This fluorescent silver staining method appears to have a high resolution. For some protein bands, the sensitivity of the fluorescent silver stain is also slightly better than that of the

fluorescent SYPRO Ruby stain. In particular, the performance of the fluorescent silver stain was improved for the ~10- to 40-kDa protein bands, indicating that the new method is particularly useful for the detection of proteins with low molecule weights. The data also suggest that the fluorescent silver stain gave a good and uniform linearity for all 14 proteins over a relatively broad range for protein quantification (**Figure 3**)[11].

In contrast to the silver nitrate stain which gave a high level of background signal and distorted peaks, the fluorescent silver stain detected the bands with a good contrast and uniform intensity distribution comparable to the SYPRO Ruby stain across all 14 proteins (**Figure 3**).

Note that insufficient washing after the gel fixation will result in high background staining (**Figure 4**). The residual acetic acid will light up the **TPE-4PA** and lead to a strong background.

FIGURE AND TABLE LEGENDS:

Figure 1: Chemical structure of TPE-4TA and its sensing mechanism to Ag^{+}. X = H or Na^{+}. Adapted from previous work, copyright 2018 WILEY-VCH[11].

Figure 2: Fluorescent silver staining. A) Summary of the protocol. **B)** A comparison of gels stained by the fluorescent silver stain (left) and SYPRO Ruby stain (right) imaged parallel with a handheld UV lamp under 365 nm irradiation. The proteins (10–200 kDa) were loaded by twofold serial dilution starting from 200–500 ng/band at the most left lane. Adapted from previous work, copyright 2018 WILEY-VCH[11].

Figure 3: Fluorescent intensity of proteins against protein amount, representative gel images, and signal profiles (from the fifth lane) of gels with three different stains. (**A**) Silver nitrate stain, (**B**) fluorescent silver stain (365-nm excitation), and (**C**) SYPRO Ruby stain. The first column of the figure shows the intensity of the stain for each band of the 14 proteins (10–200 kDa) against the amount of protein normalized to the first lane (starting on the left). The second column shows the representative gel images of the corresponding stain. The amount of protein loaded into the gel is described in **Figure 2**. Adapted from previous work, copyright 2018 WILEY-VCH[11].

Figure 4: Stained gel from a suboptimal experiment. In this experiment, the washing was insufficient after the fixation step. The residual acetic acid induced the aggregation of **TPE-4TA** and caused a strong background.

DISCUSSION:

Presented here is a novel fluorescent silver staining method for proteins in polyacrylamide gels. This strategy integrates conventional silver stains and fluorescent stains. The staining exploits the selective binding of silver ion to proteins as in other silver stains but employs a highly sensitive fluorogenic silver probe **TPE-4TA** to light up the silver bound proteins. Since the fluorogenic probe **TPE-4TA** can sense silver ions at a fairly low concentration in the nanomolar range[11], it enables a highly sensitive detection without any further demand of a reductive visualization step as needed in traditional silver stains. This will minimize run-to-run variations. Meanwhile, it avoids the use

of harsh chemicals, including formaldehyde and glutaraldehyde, which frequently interfere with peptide identification in MS analysis. Moreover, as **TPE-4TA** is AIE-active and free of any self-quenching problems, the densely accumulated dyes at a protein band can emit collectively in response to the number of dye molecules. This contributes to a wide linear dynamic range (LDR) for protein detection and a much brighter staining when compared with the SYPRO Ruby stain.

Compared with traditional silver staining, a significantly less amount of silver nitrate is required for this new staining technique—specifically, 0.0001% from 0.1% reported in commonly used silver nitrate staining protocols[4]. It can be hypothesized that the success of the new stain is purely down to the combination of the incredibly bright and sensitive fluorescent probe against a high contrast background. In comparison, reduction during the developing step in traditional silver stains requires a higher quantity of silver to produce dark visible bands, particularly when it occurs against a high background. In this method, the fluorogenic development time is longer than the traditional chemical development; however, this can be debated as a limitation or as an advantage. The gel can be left in the developing solution for long periods of time with no consequences, resulting in lower variations that may derive from the operator. Not only are there fewer steps required, but also, more pauses can be taken, which makes this protocol quite convenient. There is no risk of overstaining unlike in the traditional developing step which can overdevelop the gel, resulting in a dark background. This fluorescent silver stain is also expected to have a smaller interprotein variation, albeit a further evaluation of additional protein samples is warranted (**Figure 3**).

It is critical to follow the suggested silver nitrate concentration from the protocol; using a higher concentration does not result in better performance and sensitivity but, instead, produces a strong background fluorescence which can engulf the fluorescence of the bands. In terms of troubleshooting, loss in sensitivity and brightness may result from over-washing in the wash step prior to fluorogenic development or from insufficient silver impregnation. For this method, the fluorescence signal of the protein band is dependent on the number of protein-bound silver ions, which is essential for the formation of the fluorescent **TPE-4TA-Ag^+** complex. The protein should be saturated with silver ions to maximize the potential of this staining method.

Finally, as mentioned previously, the fluorogenic dye for silver ion used in this method, **TPE-4TA,** is also pH sensitive. A low pH can protonate the free **TPE-4TA** to fluoresce in the gel, resulting in a high background stain. Therefore, improper wash steps, which leave residual acetic acid from the fixation solution, will greatly affect the stain quality. It is important to keep the gel relatively neutral in pH in the fluorogenic development step (**Figure 4**). It could also be helpful to adjust the pH of the **TPE-4TA** solution before staining. It is expected that this dye will be commercialized in the near future.

In summary, we described a practical protocol of the novel fluorescent silver stain for the visualization of total proteins in SDS-PAGE gel. The staining is straightforward, easy-to-use, cost-efficient, and has a good staining performance. This method can greatly facilitate the identification of the protein bands in gels and provides a useful tool for protein analysis.

ACKNOWLEDGMENTS:

The authors would like to thank Patrick Chan at the Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet, for his technical support. S. X. is grateful for the support from the Swedish Research Council (Grant No. 2017-06344).

DISCLOSURES:

A patent application on this fluorescent silver staining has been filed.

REFERENCES:

1. Chevalier, F. Highlights on the capacities of "Gel-based" proteomics. *Proteome Science.* **8** (1), 23 (2010).

2. Harris, L. R., Churchward, M. A., Butt, R. H., Coorssen, J. R. Assessing detection methods for gel-based proteomic analyses. *Journal of Proteome Research.* **6** (4), 1418-1425 (2007).

3. Gauci, V. J., Wright, E. P., Coorssen, J. R. Quantitative proteomics: assessing the spectrum of in-gel protein detection methods. *Journal of Chemical Biology.* **4** (1), 3-29 (2011).

4. Candiano, G., Bruschi, M. *et al*. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis.* **25** (9), 1327-1333 (2004).

5. Chevallet, M., Luche, S., Rabilloud, T. Silver staining of proteins in polyacrylamide gels. *Nature Protocols.* **1** (4), 1852-1858 (2006).

6. Jin, L. T., Hwang, S. Y., Yoo, G. S., Choi, J. K. Sensitive silver staining of protein in sodium dodecyl sulfate-polyacrylamide gels using an azo dye, calconcarboxylic acid, as a silver-ion sensitizer. *Electrophoresis.* **25** (15), 2494-2500 (2004).

7. Celis, J. E. *et al*. *Cell Biology: A Laboratory Handbook.* Elsevier Academic Press. Amsterdam, the Netherlands (2006).

8. Bartsch, H., Arndt, C., Koristka, S., Cartellieri, M., Bachmann, M. Silver Staining Techniques of Polyacrylamide Gels. *Methods in Molecular Biology.* **869** (1), 481-486 (2012).

9. Rabilloud, T., Vuillard, L., Gilly, C., Lawrence, J. Silver-staining of proteins in polyacrylamide gels: a general overview. *Cellular and Molecular Biology.* **40** (1), 57-75 (1994).

10. Zhou, M., Yu, L. Proteomic Analysis by Two-Dimensional Polyacrylamide Gel Electrophoresis. *Advances in Protein Chemistry.* **65** (1), 57-84 (2003).

11. Xie, S. *et al.* Fluorogenic Ag^{+}-Tetrazolate Aggregation Enables Efficient Fluorescent Biological Silver Staining. *Angewandte Chemie International Edition.* **57** (20), 5750-5753 (2018).

12. Protein gel electrophoresis technical handbook.

https://www.thermofisher.com/content/dam/LifeTech/global/Forms/PDF/protein-gel-electrophoresis-technical-handbook.pdf. Thermo Fisher Scientific (2015).

Figure 1

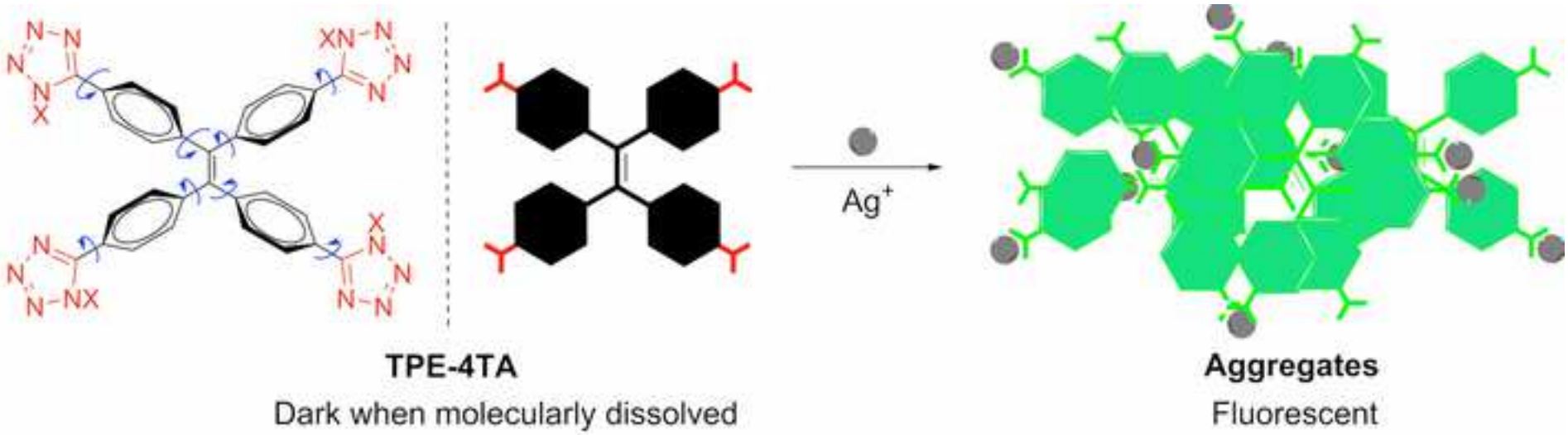
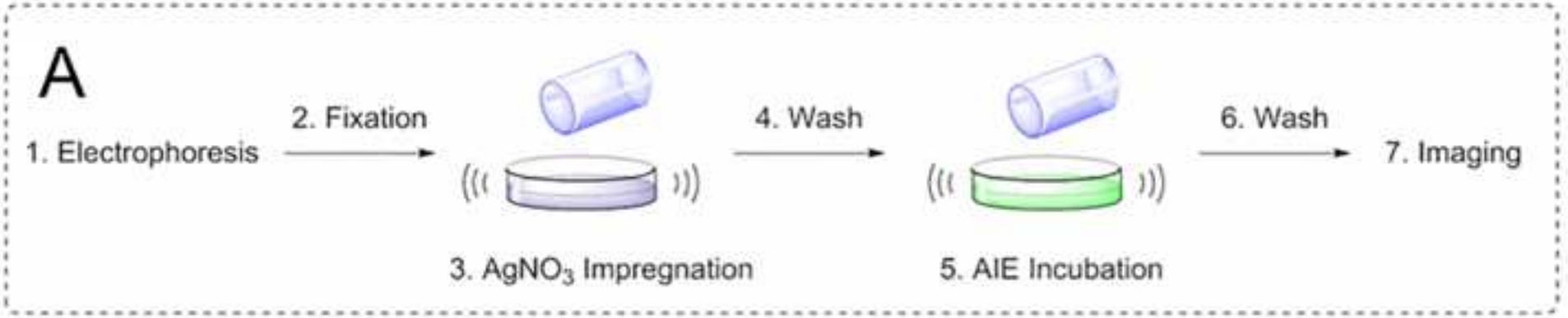


Figure 2



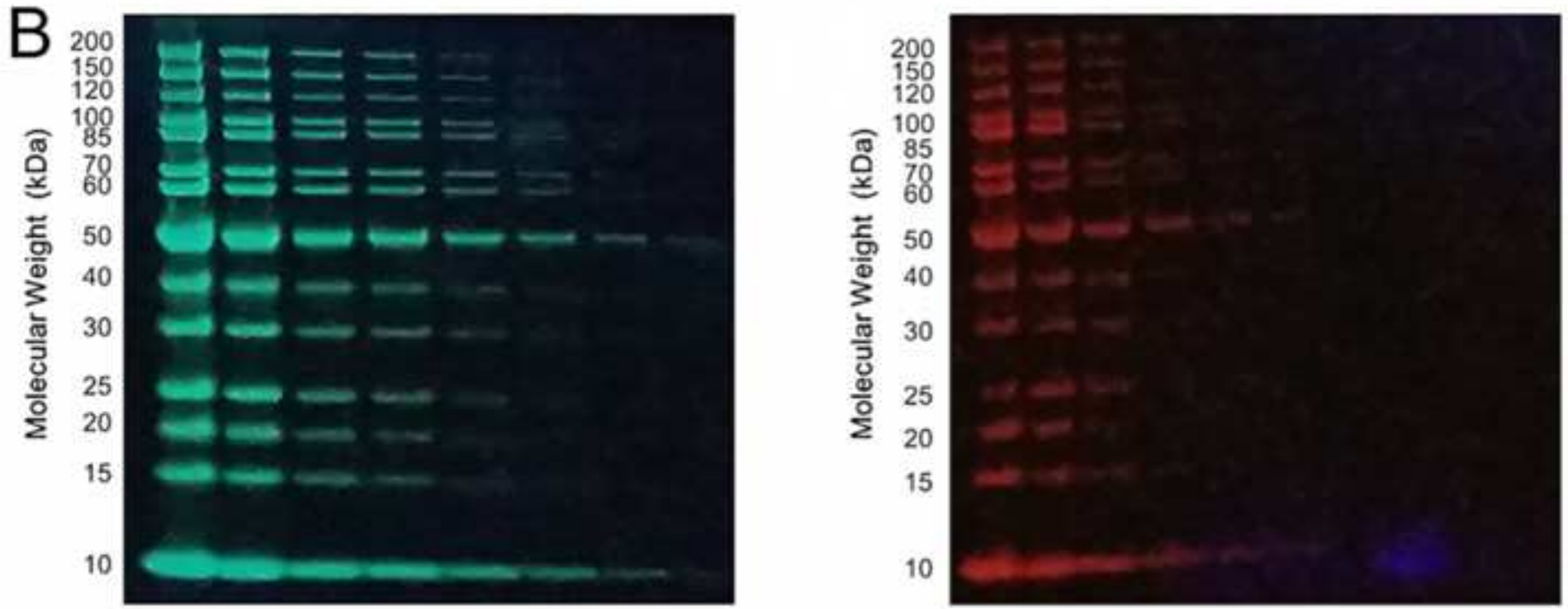


Figure 3

Click here to access/download;Figure;Figure 3.psd

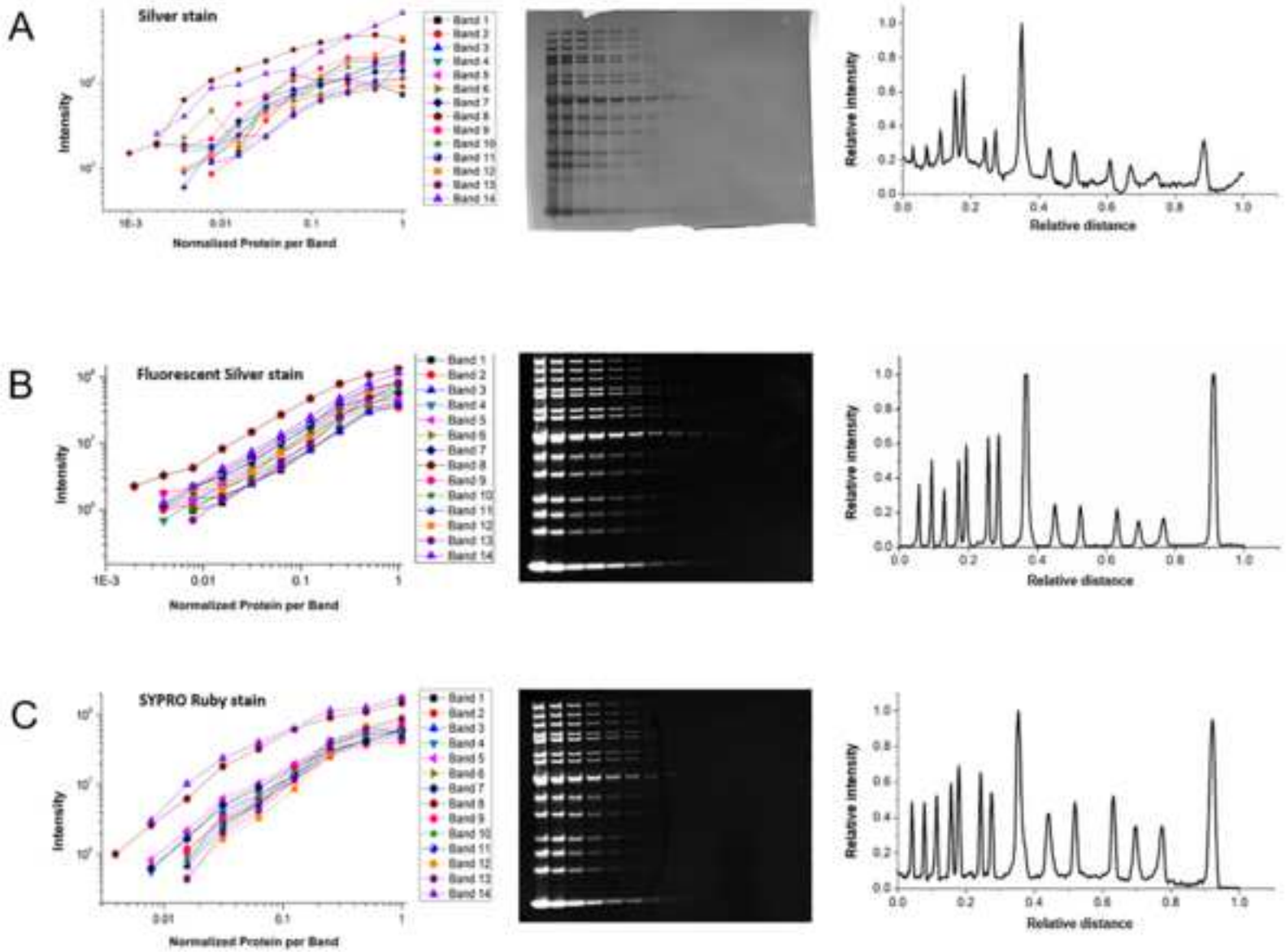


Figure 4

Click here to access/download;Figure;figure 4.psd

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
LDS Sample Buffer (4X)	Thermo Fisher Scientific	NP0007	Reagent
4-12% Bis-Tris Protein Gels, 1.0 mm, 15-well	Thermo Fisher Scientific	NP0323BOX	Precast gel
Sample Reducing Agent (10X)	Thermo Fisher Scientific	NP0004	Reagent
MES SDS Running Buffer (20X)	Thermo Fisher Scientific	NP0002	Reagent
Mini Gel Tank	Thermo Fisher Scientific	A25977	Equipment
300W Power Supply (230 VAC)	Thermo Fisher Scientific	PS0301	Equipment
Unstained Protein Ladder	Thermo Fisher Scientific	26614	Sample
Silver nitrate	Sigma-Aldrich	31630-25G-R	Reagent
Ethanol	Bragg and co.	42520J	Reagent
Acetic acid	J.T. Baker	103201A	Reagent
Milli-Q Synthesis A10	Merk	-	Provides 18.2 MΩ.cm water
gel documentation system (c600 model)	Azure biosystems	-	Equipment

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: FLUORESCENT SILVER STAINING OF PROTEINS IN POLYACRYLAMIDE GELS

Author(s): ALEX WONG, SHENG XIE, BEN ZHONG TANG, SIJIE CHEN

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	SIJIE CHEN
Department:	MING WAI LAU CENTRE OF REPARATIVE MEDICINE
Institution:	KAROLINSKA INSTITUTET
Title:	ASSISTANT PROFESSOR
Signature:	Chen Sijie
Date:	1/8/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Sijie Chen, Ph.D.
Assistant Professor
Ming Wai Lau Centre for Reparative Medicine
Karolinska Institutet
Hong Kong
Email: Sijie.chen@ki.se
Tel: +852-26243811

Dr. Phillip Steindel
Review Editor, *JoVE*
1 Alewife Centre, Suite 200
Cambridge, MA 02140
Tel: 617.674.1888

3rd Aug.2018

MS ID: **JoVE58669**
Subject: **Invited Video-Article**
MS Title: **Fluorescent Silver Staining of Proteins in Polyacrylamide Gels**
MS Authors: **Alex Wong, Sheng Xie, Ben Zhong Tang and Sijie Chen***

Dear Dr. Phillip Steindel,

Thank you very much for your email on 24 Jul. 2018 and for forwarding us the reviewers' comments and suggestions. We have revised our manuscript according to all the comments. All the modifications are tracked in the revised manuscript. Given below are our specific point-by-point responses to the reviewers' comments.

Response to your comments

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

   The manuscript has been proofread to correct any spelling or grammar issues. The spelling has been made consistent with standard American English.

2. *Please expand the Summary (10-50 words total) to briefly describe the applications of this protocol.*

   We have expanded the summary to briefly describe the applications of this protocol as requested: "Here, we describe a detailed protocol which outlines a new fluorescent staining technique for total protein detection in polyacrylamide gels. The protocol utilizes a silver ion specific fluorescence turn-on probe, which detects the Ag^+ -protein complex. This technique makes use of the specific bio-affinity of silver ions to proteins, but eliminates certain limitations surrounding traditional silver stains."

3. Please spell out each abbreviation the first time it is used.

Correction have been made for abbreviations to be spelt out for its first use such as; Sodium Dodecyl Sulphate-Polyacrylamide-Gel Electrophoresis (SDS-PAGE), mass spectrometry (MS), Ultra-violet (UV) and Lithium Dodecyl Sulphate (LDS).

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Invitrogen, Thermos Fisher Scientific, Millipore, PowerEase, NUPAGE, Azurespot, etc.

Thank you for the reminder, the trademark symbols and names have been removed from the manuscript and table of materials in the revision.

5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The personal pronouns have now been removed in the revised protocol. "The dye TPE-4TA was synthesized following the protocol that was recently reported.[10]"

6. 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. Please move the discussion about the protocol to the Discussion.

We have edited the words to the suggested action items in the revision and removed certain phrases as mentioned above.

7. Lines 84-91: Please revise this paragraph to discrete numbered steps.

We have revised the brief description of conditions for sample preparation and electrophoresis into discrete numbered steps.

8. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

We have revised the 'Notes' as suggested.

9. 3.2: Please specify how to adjust the pH of the solution.

The revision now specifies how to check and adjust the pH of the solution: "To prepare 100 mL of the fluorogenic developing solution (10 μM), add 10 mL of the **TPE-4TA** stock solution into 90 mL ultra-pure water. Check the pH of the solution using a pH meter and tune to 7-9 using acetic acid (1 mM) or sodium hydroxide (1 mM)."

10. *3.3: Please specify the incubation temperature.*

The incubation temperature is at room temperature and is specified in the revision.

11. *4.2: What volume of water is used to rinse? How many times?*

The gel is rinsed in 100 mL of water once, for a period of 5 min.

12. *4.3: Please add more details about how to image the gel.*

The gel can be visualized with any UV lamp containing commercial gel documentation machine at the 302 nm and 365 nm channel. This has been specified more clearly in the revision.

13. *Discussion: Please also discuss critical steps within the protocol and any limitations of the technique.*

The critical step in the discussion includes the adjustment of pH and preventing the gel from acid exposure. Furthermore, an additional critical information within the protocol has been discussed, which is to follow the suggested silver nitrate concentration in the protocol as any higher may result in high background staining. Other limitations of the technique to be included in the revision is the fact that the dye is not widely commercially available and that the AIE development takes longer than a conventional chemical development.

14. *References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.*

The journal titles have now been corrected in the revision with the volume and issue numbers included.

15. *Please follow the book citation example below to reformat book references: Kioh, L.G. et al. Physical Treatment in Psychiatry. Blackwell Scientific Pubs. Boston (1988).*

Thank you for your suggestion, reference [6] now follows the example provided. Celis, J. E. et al. Cell Biology: A Laboratory Handbook. Elsevier Academic Press. Amsterdam (2006).

16. *Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*

Thank you for the reminder, trademarks, symbols and commercial names have been removed from the Table of Equipment and Materials.

Response to the Comments and Suggestions of Reviewer 1

1) *data suggest that apparently Sypro can stain proteins better than Silver staining, which is not normally true, of course it depends on the quality of silver staining, for example if silver stain is conducted by a nice protocol and properly or for example with Blue silver (see Electrophoresis. 2004 May;25(9):1327-33, that should be at least cited along with and its advantages/disadvantages compared to the method proposed) it is definitely more sensitive. The only real advantage of the proposed method, apart from its being fluorescent, is that it's fast and intrinsically displaying very low background.*

   We fully agree with the comment regarding the SYPRO stain and silver stain. There are many silver staining protocols. In this study we used a simple silver nitrate stain, which has minimal steps and can be further modified for the silver impregnation step of the fluorescent silver stain. This serves as a control and benchmark for our fluorogenic silver stain to see how much the AIE dye can improve the performance in terms of sensitivity and contrast, when combined with the silver ions. This allows direct observation of how much the performance can improve with the AIE fluorophore without any extraneous influence from other reagents.

   There are numerous silver staining protocols to compare. In particularly when a sensitization step is applied, the detection of silver staining can be much improved. We also noticed the highly sensitive blue silver method, which is now cited in the revised version. Basically, we also prefer a silver nitrate staining protocol variant in the absence of an aldehyde sensitizer since it may lead to a stain compatible with mass spectroscopy.

2) *Another drawback is the need for a particular device for the reading, anyway this should not be a problem.*

   Yes, this is true for many fluorescence-based techniques. In our case, it is simple because many gel imagers have UV channels and thus can be used directly to read the gel.

Response to the Comments and Suggestions of Reviewer 2

1) *The control method to which the new method has been compared, is not adequate. There are many other variants of silver staining that are one order of magnitude more sensitive than the one selected. It is as if someone describing a new staining method with a dye would compare it to old style Coomassie blue with alcohol-acid de-staining and not to modern colloidal Coomassie blue.*

   Thank you for your comment. There are many silver staining protocols. In particular when a sensitization step is used, the quality can be improved highly. For the same protocol, the staining quality will also vary from operator to operator. There is no silver staining protocol

that can be claimed as the best for use as a standard silver staining method for comparison. Regarding the sensitivity, our method cannot exceed the detection limit of the best silver stain in this sample (the reported LOD is lower than many of the paper reporting highly sensitive silver staining method). That is why the SYPRO method is also used in parallel as a control group. SYPRO is a widely used fluorescent stain, and its performance has been fully compared with many silver stains.

In this work, we want to highlight the use of "fluorogenic development", instead of the classic chemical development which reduces the silver ions. Taking this into account, the control method we have selected is fair to compare. Furthermore this allows us to understand the difference in the performance between our novel method and the basic fundamental silver staining method. We selected this protocol variant of the silver stain, specifically because it uses silver nitrate and avoids many harmful chemicals such as ammonia and glutaraldehyde without compromising MS compatibility.

Taking this into account, our selected silver protocol is a suitable control regarding the design of our staining strategy. Many other silver stains, in particular the sensitive ones, can be good alternatives to compare the detection limits in a fair way. Meanwhile, we adopt the standard SYPRO fluorescent stain as a suitable control to discuss about the sensitivity in the detection of proteins.

2) *The organic probe which is absolutely mandatory for the method is not widely available. Most of the end users do not have the chemical knowledge to prepare it or the money to buy a custom synthesis.*

At the time of writing, the AIE probe we have described is not yet widely available, however the probe is patented and will be commercialized very soon.

Please let me know if further information is required.

Yours sincerely,

Sijie Chen, Ph.D.
Assistant Professor
Karolinska Institutet

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Jun 25, 2018

This Agreement between Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet, Hong Kong -- Alex Wong ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4375830600558
License date	Jun 25, 2018
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Angewandte Chemie International Edition
Licensed Content Title	Fluorogenic Ag –Tetrazolate Aggregation Enables Efficient Fluorescent Biological Silver Staining
Licensed Content Author	Sheng Xie, Alex Y. H. Wong, Ryan T. K. Kwok, et al
Licensed Content Date	Apr 14, 2018
Licensed Content Volume	57
Licensed Content Issue	20
Licensed Content Pages	4
Type of use	Journal/Magazine
Requestor type	Author of this Wiley article
Is the reuse sponsored by or associated with a pharmaceutical or medical products company?	no
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	3
Original Wiley figure/table number(s)	Figure 1,3,4 and S10
Will you be translating?	No
Circulation	5000
Title of new article	Fluorescent Silver Staining of Proteins in polyacrylamide gels
Publication the new article is in	JoVE
Publisher of new article	MyJove Corp
Author of new article	Alex. Y.H. Wong
Expected publication date of new article	Nov 2018
Estimated size of new article (pages)	6
Requestor Location	Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet, Hong Kong Units 608-613 Building 15 Science Park 15W Science Park W Ave Sha Tin, 000 Hong Kong

	Attn: Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet, Hong Kong
Publisher Tax ID	EU826007151
Total	0.00 USD

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.

- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order,** is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner.**For STM Signatory Publishers clearing permission under the terms of the STM Permissions Guidelines only, the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts,** You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or

to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.

- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC)License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library

http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.10 Last updated September 2015

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.