Video Article

Fluorescent Silver Staining of Proteins in Polyacrylamide Gels

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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. 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.
2. Dilute the samples with a mixture of distilled water, lithium dodecyl sulfate (LDS) buffer, and a sample-reducing agent.
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).
4. Run the gel at a constant voltage of 200 V for 30 min.

2. Fixation of the Gel

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. 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

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

4. Fluorogenic Development of the Gel

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.
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.
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. 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

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

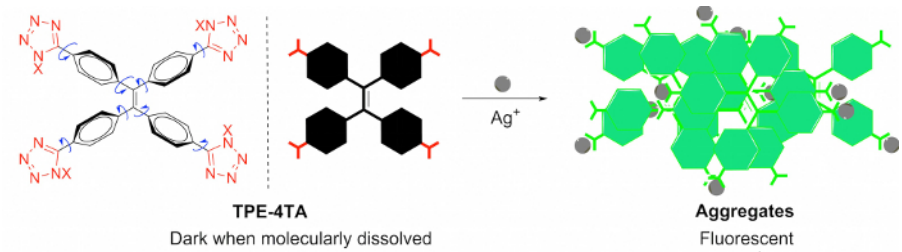

Figure 1: Chemical structure of TPE-4TA and its sensing mechanism to Ag^+. X = H or Na^+. Adapted from previous work, copyright 2018 WILEY-VCH[11]. Please click here to view a larger version of this figure.

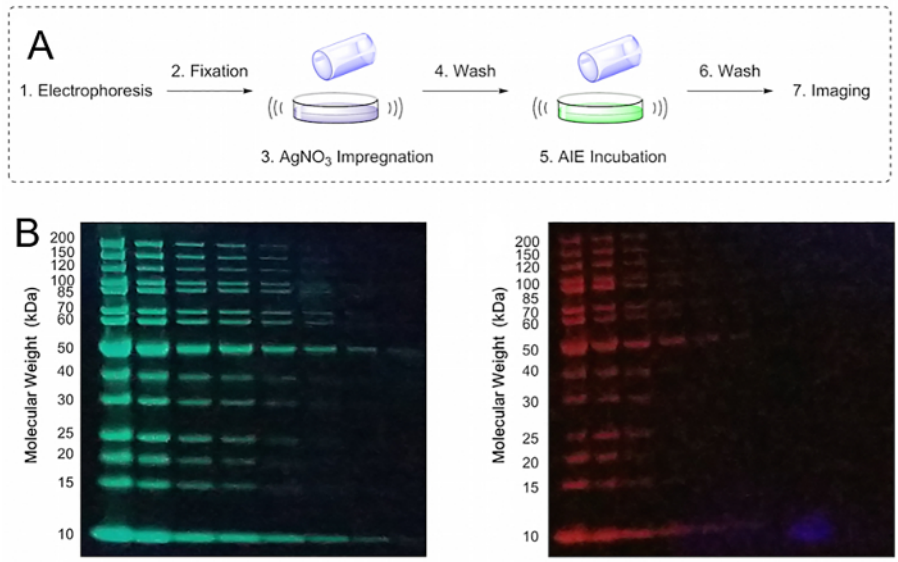

Figure 2: Fluorescent silver staining. A) Summary of the protocol. **B)** A comparison of gels stained by the fluorescent silver stain (left) and SYPRO Ruby stain (right) imaged parallel with a handheld UV lamp under 365 nm irradiation. The proteins (10-200 kDa) were loaded by twofold serial dilution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

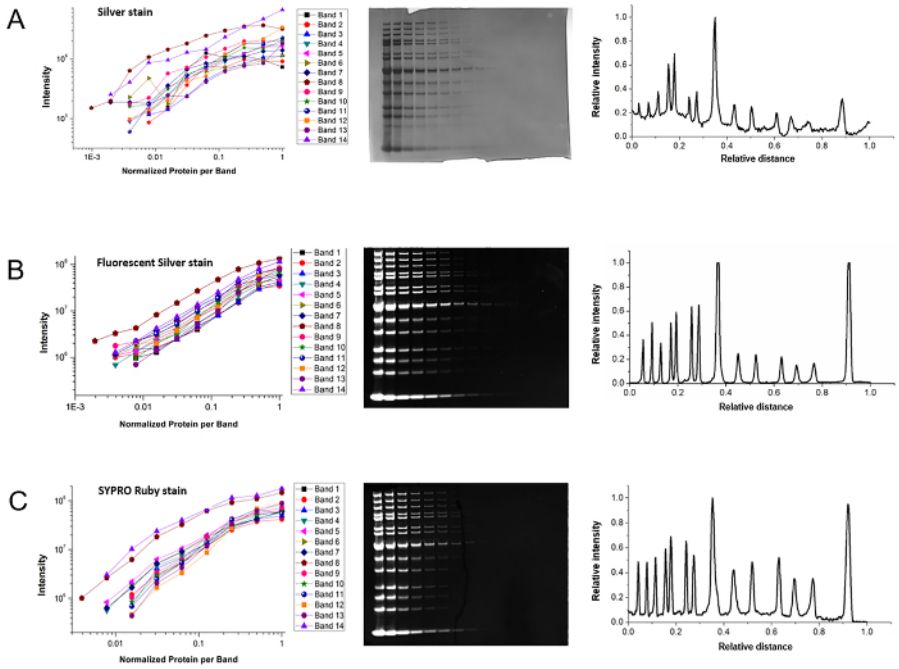

Figure 3: Fluorescent intensity of proteins against protein amount, representative gel images, and signal profiles (from the fifth lane) of gels with three different stains. (**A**) Silver nitrate stain,(**B**) fluorescent silver stain (365-nm excitation), and (**C**) SYPRO Ruby stain. The first column 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]. Please click here to view a larger version of this figure.

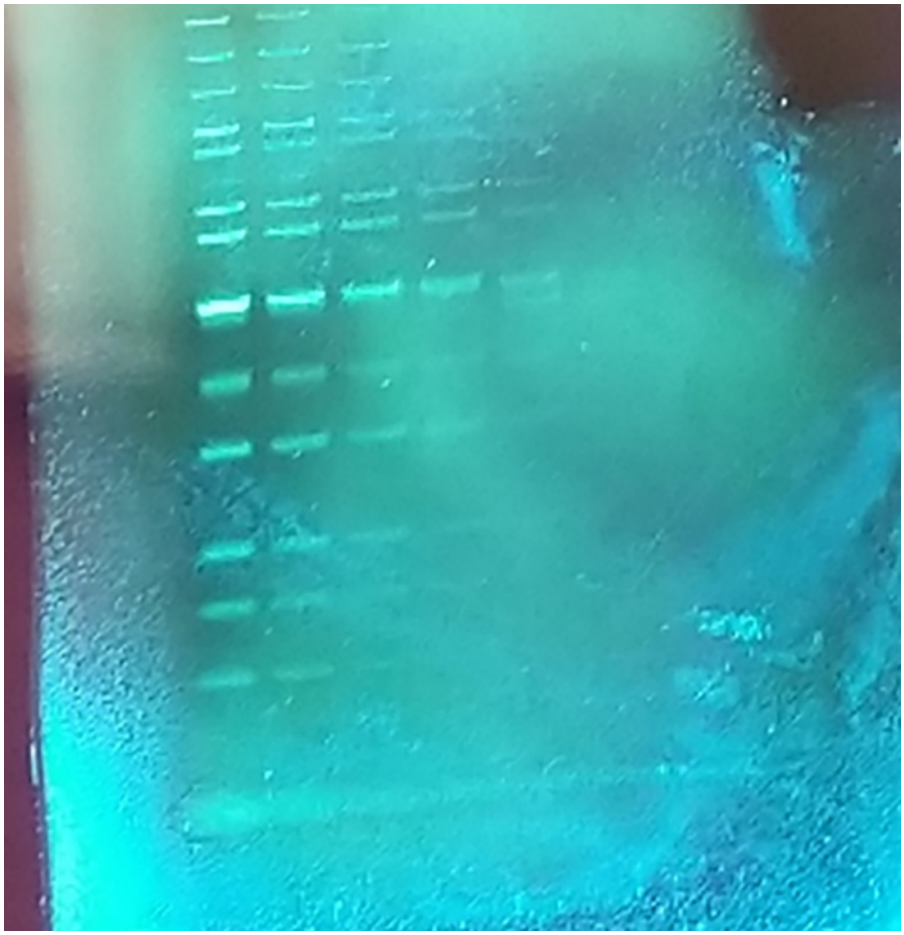

Figure 4: Stained gel from a suboptimal experiment. In this experiment, the washing was insufficient after the fixation step. The residual acetic acid induced the aggregation of **TPE-4TA** and caused a strong background. Please click here to view a larger version of this figure.

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.

Disclosures

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

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