Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58470R2
Full Title:	Separation of spinach thylakoid complexes by Green Gel electrophoresis followed by band characterization using Time Correlated Single Photon Counting
Keywords:	Native Green Gel; Complex; Supercomplex; Megacomplex; TCSPC; Fluorescence; Thylakoid; Photosystem
Corresponding Author:	Eliezer Moses Schwarz Lansing Community College Mason, MI UNITED STATES
Corresponding Author's Institution:	Lansing Community College
Corresponding Author E-Mail:	eliezer.schwarz@gmail.com
Order of Authors:	Eliezer Schwarz
	Gary Blanchard
Additional Information:	
Question	**Response**
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TITLE:

Separation of Spinach Thylakoid Protein Complexes by Native Green Gel Electrophoresis and Band Characterization using Time-Correlated Single Photon Counting

AUTHORS AND AFFILIATIONS:

Eliezer Schwarz[1], Gary Blanchard[2]

[1]Department of Plant Biology, Michigan State University, East Lansing, MI, USA
[2]Department of Chemistry, Michigan State University, East Lansing, MI, USA

Corresponding Author:

Eliezer Schwarz (Eliezer.Schwarz@star.lcc.edu)
Tel: (309) 212 5766

Email Address of Co-Author:

Gary Blanchard (Blanchard@chemistry.msu.edu)

KEYWORDS:

Native gel electrophoresis, green gel, thylakoid, protein complex, supercomplex, photosystem, time-correlated single photon counting (TCSPC)

SUMMARY:

Here we present a protocol to separate solubilized thylakoid complexes by Native Green Gel electrophoresis. Green gel bands are subsequently characterized by Time Correlated Single Photon Counting (TCSPC) and basic steps for data analysis are provided.

ABSTRACT:

The light reactions of photosynthesis are carried out by a series of pigmented protein complexes in the thylakoid membranes. The stoichiometry and organization of these complexes is highly dynamic on both long and short time scales due to processes that adapt photosynthesis to changing environmental conditions *(i.e.,* non-photochemical quenching, state transitions, and the long-term response). Historically, these processes have been described spectroscopically in terms of changes in chlorophyll fluorescence, and spectroscopy remains a vital method for monitoring photosynthetic parameters. There are a limited number of ways in which the underlying protein complex dynamics can be visualized. Here we describe a fast and simple method for the high-resolution separation and visualization of thylakoid complexes, native green gel electrophoresis. This method is coupled with time-correlated single photon counting for detailed characterization of the chlorophyll fluorescence properties of bands separated on the green gel.

INTRODUCTION:

Photosynthetic organisms must constantly adjust their physiology to changing environmental conditions to maximize their productivity and successfully compete with neighbors[1]. This is especially true of the machinery responsible for the light reactions of photosynthesis, as ambient

light conditions can fluctuate by three orders of magnitude between shadows and full sunlight. Additionally, environmental factors such as drought, cold, or heat stress can reduce the availability of carbon dioxide for carbon fixation, which is the natural electron sink for the products of the light reactions. Plants must, therefore, harvest and utilize solar radiation as efficiently as possible while retaining the ability to dissipate excess light energy as necessary. While photooxidative damage still occurs routinely under all light conditions[2,3], failure to manage absorbed excitation energy successfully can lead to the catastrophic cell damage and death. Several adaptive mechanisms exist that allow the photosynthetic apparatus to be tuned both to changes in prevailing environmental conditions and to transient fluctuations (*i.e.,* over both long and short timescales)[4]. These include the long-term response (LTR) and non-photochemical quenching (NPQ). NPQ is itself considered to encompass at least three other component phenomena, including state transitions (qT), rapidly inducible energy quenching (qE), and photoinhibition (qI)[5].

These processes were originally observed and defined largely in terms of spectroscopic phenomena [*e.g.,* NPQ refers to a drop in observed chlorophyll fluorescence (quenching of chlorophyll fluorescence) that is not due to an increase in the rate of photochemistry][6]. The term "state transitions" similarly refers to the observed change in the relative amount of fluorescence from PSI and PSII[7]. While the spectroscopic techniques that have made enumeration of these phenomena possible [in particular, pulse amplitude modulated (PAM) fluorescence spectroscopy] and continue to be a vital means for observing and dissecting photosynthetic processes *in vivo*, a great deal of biochemistry is required to elucidate the mechanisms underlying these spectroscopic observations. State transitions, for instance, involves a phosphorylation/dephosphorylation cycle of the LHCII proteins by the STN7 kinase and TAP38/PPH1 phosphatase, respectively[8-10]. This cycle adjusts the physical distribution of the LHCII antenna between the two photosystems by moving a portion of LHCII trimers from PSII to PSI, thereby changing the absorption cross section of the photosystems[11,12]. The qE component of NPQ rapidly converts excess excitation energy into heat through the actions of the violaxanthin/zeaxanthin epoxidation/de-epoxidation cycle and the PsbS protein. The exact role of PsbS in this process is still not fully understood[13]. The qI component of NPQ, photoinhibition, is generally ascribed to damage to the D1 protein of PSII. Restoration of full photosynthetic competence requires an elaborate repair process to fix damaged PSII photocenters. The PSII repair cycle involves the migration of PSII complexes out of the granal stacks, dismantling of the complexes, replacement of damaged D1 proteins, reassembly of the PSII complexes, and movement of PSII complexes back into the granal stacks[14]. The exact nature of photoinhibition and PSII photodamage remains a subject of intense scrutiny[15].

The difficulty in studying phenomena like state transitions or PSII repair arises in part from the fact that there is not one simple way to visualize the mechanics of complex biochemical systems. The classic biochemical approach to understanding a process is to first separate its components so that they can be characterized in isolation. Native gel electrophoresis arose from successful efforts in the 1980s to separate and characterize the photosystem complexes from the thylakoid membranes with more preparative methods (namely sucrose gradient centrifugation and chromatography)[16]. The detergent systems developed to gently solubilize the native complexes

from the thylakoid membranes were soon adapted to electrophoretic separation methods, most notably by Allen and Staehelin[17] and and Peter and Thornber[18], giving rise to native green gel electrophoresis. While representing only one out of a variety of techniques in the experimental arsenal, native PAGE has a number of attractive characteristics that have made it a widely employed method in photosynthesis research. Native PAGE is relatively fast and simple, requiring little specialized equipment, while providing high resolution separation of a large number of thylakoid complexes simultaneously. This makes native PAGE a convenient tool for studying thylakoid dynamics and, when combined with standard PAGE in the second dimension as well as a variety of detergent and buffer systems, a versatile system for finding and characterizing new thylakoid complexes.

That being said, native green gels have had a reputation for being an unreliable technique, especially in inexperienced hands, as it is easy to produce poor results consisting of fuzzy, smeary gels with few bands. This problem was solved, in part, with the introduction of blue-native PAGE[19]. The use of coommassie dye in the BN buffer system makes protein separation more robust. Therefore, BN-PAGE is often an easier and more reliable technique for a relative novice to set up and can provide high resolution separations of thylakoid complexes. For these reasons, BN-PAGE has become the method of choice for most work of this field. While BN-PAGE is generally slower to run than green gel electrophoresis, its main drawback is that the coommassie dye staining interferes with the identification of faint chlorophyll-containing bands, while also making downstream spectroscopic characterization problematic.

The biochemical information provided by native gels and 2D SDS-PAGE can be greatly strengthened when combined with data from spectroscopic techniques. Regardless of the system employed, a central problem with using native gels to identify complexes is that the identification can always be challenged (*i.e.,* the proteins found in a band could always represent comigrating complexes or components, rather than a single physiologically authentic complex). Spectroscopic characterization provides biophysical information about the pigments in green gel bands and can be used to determine what types of complexes they are likely to contain. Chlorophyll fluorescence is especially useful in this regard due to the often dramatically different spectra and fluorescence lifetimes that are characteristic of different photosynthetic pigment-protein complexes. While simple steady-state 77K fluorescence spectra have historically been useful in confirming the identities of native gel complexes, modern time-correlated single photon counting (TCSPC) can provide much more information. TCSPC allows not only the characterization of complexes based on fluorescence lifetimes, but also makes possible the detailed description of energy transfer between spectral components within a complex. This kind of characterization is becoming increasingly necessary as the use of various native gel systems spreads and new putative complexes are discovered, allowing the identification of protein complexes to be better authenticated and providing new biophysical information about how these complexes work.

In this paper we provide a method that allows those having little or no experience with native gel electrophoresis to achieve high quality resolution of native thylakoid complexes for the purpose of investigating the mechanics of the light reactions of photosynthesis. This basic technique can

then be augmented at the experimenter's discretion to improve results or extend applicability to other species. We then describe the process for subjecting native green gel bands to TCSPC, as well as some steps for basic analysis and presentation of the data provided by the technique. The coupling of native gel electrophoresis with TCSPC analysis extends the utility of these gel systems by providing authentication and biophysical characterization of protein complexes within the bands. The green gel system described here is based on that developed by Allen and Staehelin[17] with some modifications and is the same as that used in Schwarz *et al*.[20]. This system is one of many but has specific features that are useful for this methodology. It is rapid enough so that thylakoid isolation, gel electrophoresis, and TCSPC analysis convenient can be performed in one day, obviating potential problems of sample storage and degradation. We also find that this method is robust in the hands of inexperienced users, while still providing results that range from good to superior, depending on the degree of optimization.

It is important to bear in mind that the complexes visualized on a native gel depend on both the detergent and buffer systems used, as well as on the biology of the organism under investigation. Different detergent and buffer systems preferentially separate different kinds of complexes, and a given photosynthetic organism will have different complexes from other organisms, not all of which will be present under any given circumstance. The system described here is particularly suited to the study of PSI megacomplexes, as described in Schwarz *et al*.[20], but it falls on the more destabilizing end of the spectrum for those studying PSII megacomplexes. For a comprehensive study of the various detergent and buffer systems used in native gel electrophoresis of thylakoid proteins, it is recommended to review Järvi *et al*.[21] and Rantala *et al.*[22].

PROTOCOL:

1. Stock Solutions Preparation for Pouring Native Green Gels

1.1. Prepare a 4x concentrated buffer solution for resolving gels consisting of 40% glycerol, 200 mM glycine, and 100 mM Tris buffered to pH 8.3.

1.2. Prepare a 4x concentrated buffer solution for stacking gels consisting of 40% glycerol, 200 mM glycine, and 100 mM Tris buffered to pH 6.3.

1.3. Store these buffers at 4 °C to prevent the growth of mold.

Note: The buffers are stable for months at 4 °C, so preparation of 100-200 mL of each buffer for continued use is recommended.

1.4. Prepare 1 L of 10x running buffer containing 250 mM Tris HCl pH 8.3, 1.92 M glycine, and 1% SDS. Store the 10x running buffer on the benchtop.

2. Stock Solution Preparation for Isolation and Solubilization of Thylakoids

2.1. Prepare 100 mL of TMK homogenization buffer containing 50 mM Tris buffer (pH 7), 10 mM

$MgCl_2$, and 10 mM KCl, and store at 4 °C.

Note: This is the main buffer for homogenizing and manipulating thylakoid samples. Alternatively, concentrated stock solutions can be prepared and diluted as necessary (Tris buffer, $MgCl_2$, and KCl can all be easily stored on the benchtop as 1 M concentrates).

2.2. Prepare stock solutions of the thylakoid solubilization detergents, B-decyl maltoside (DM) and n-octyl B-d-glucoside (OG), by dissolving each detergent in TMK buffer at 20% w/v. Freeze at -20 °C in 1 mL aliquots.

2.3. Prepare thylakoid solubilization buffer (SB), which is also the sample loading buffer.

2.3.1. First, make TMK-glycerol buffer by combining 7 mLs of TMK buffer and 3 mLs of glycerol.

2.3.2. To 800 μL of TMK-glycerol buffer, add 100 μL of DM stock solution and 100 μL of OG stock solution. Store this SB working solution, containing 2% DM and 2% OG, frozen at -20° C in 1 mL aliquots.

Note: Each aliquot can be thawed and refrozen as needed.

3. **Pouring Green Mini Gels for Later Use**

3.1. Prepare separate stacking and resolving gel solutions in 15 mL disposable test tubes.

Note: The volumes provided are sufficient for a single mini gel using 1.5 mm plate spacers.

3.1.1. To make the stacking gel solution, combine 1.25 mL of 4x stacking gel buffer, 0.5 mL of 40% acrylamide stock solution (39:1 C), and 3.25 mL of water to give 5 mL of 4% acrylamide in 1x stacking buffer. To make the resolving gel solution combine 1.875 mL of 4x resolving buffer, 0.94 mL of 40% acrylamide stock solution (39:1 C), and 4.7 mL of water to give 7.5 mL of 5% acrylamide in 1x resolving buffer.

3.2. Pour the resolving gel.

3.2.1. Add 50 μL of 10% ammonium persulfate (APS) to the resolving gel solution, then add 10 μL of TEMED, cap the tube, and gently invert several times to mix. Immediately pour the gel solution between the gel plates, leaving approximately 1 cm between the top of the resolving gel and bottom of the comb teeth for the stacking gel.

3.2.2. Gently pipette 100% ethanol onto the top of the resolving gel to level the gel.

Note: If the gel does not set within 15 min, fresh APS solution should be made and/or new TEMED should be used.

3.2.3. After the gel has polymerized (the interface between the gel and the ethanol will be readily visible and will not move when the gel is tipped), pour off the ethanol and blot with an absorbent paper.

3.3. Pour the stacking gel.

3.3.1. Add 25 µL of 10% APS to the stacking gel solution, add 5 µL of TEMED, then cap and invert to mix in the same manner as the resolving gel. Pour the gel solution on top of the resolving gel until the space between the plates is completely filled and insert a 10-well comb.

Note: When ready to be used, remove the comb from the gel and rinse the wells with water, making sure that the wells are straight and unobstructed by gel. The gel can be stored with the comb in place at 4 °C for at least several days.

4. **Isolation of Crude Thylakoid Membranes from Spinach Leaves**

Note: All steps should be carried out on ice using pre-chilled equipment and buffers. Dim lighting is also recommended. Depending on the experimenter's discretion and the biological processes under study, protease and/or phosphatase inhibitors should be added fresh to TMK buffers before homogenization.

4.1. Completely homogenize spinach leaves in TMK buffer with a glass Dounce homogenizer.

Note: Approximately 1 to 2 mL of buffer is normally sufficient for a small baby spinach leaf. A single baby spinach leaf can generally provide enough material to load several wells on a 1.5 mm mini gel.

4.2. Filter the crude leaf homogenate to remove insoluble debris.

4.2.1. To make a simple filtering device, cut a delicate task wipe in half and fold it into quarters. Pack the delicate task wipe into the bottom of a 5 mL disposable syringe and pre-wet the wipe with TMK buffer.

4.2.2. Use the syringe plunger to press excess buffer out of the delicate task wipe and be sure that the wipe filter is pressed firmly to the bottom of the syringe after the plunger is removed.

4.2.3. Pipette the leaf homogenate onto the center of the wipe filter and use the plunger to pass the homogenate through the filter. Collect the filtered homogenate in a 1.5 mL centrifuge tube.

4.3. Centrifuge the homogenate at 5,000 x g for 10 min at 4° C to pellet insoluble material, including thylakoid membranes. Discard the supernatant and resuspend the pellet in 1 mL of TMK buffer.

4.4. Normalize the amount of chlorophyll in each sample by adjusting the volume of each

resuspended thylakoid sample so that each sample contains the same total amount of chlorophyll, as described below.

Note: This will allow each sample to be solubilized in the same volume of detergent and minimizes variability in solubilization due to differences in pellet volume.

4.4.1. To extract chlorophyll from each sample of resuspended thylakoid membranes, take a 50 µL aliquot in a 1.5 mL microcentrifuge tube and add 950 µL of methanol to it. Cap the tube and mix by inverting several times.

4.4.2. Centrifuge the methanol/chlorophyll extract at 10,000 x g for 10 min to pellet precipitated proteins.

4.4.3. Determine the chlorophyll concentration of the pigment containing supernatant according to Porra *et al*.[23]. Take absorbance readings at 652 and 665 nm using a spectrophotometer and a 1 cm cuvette. Determine total chlorophyll concentration using the equation below:

$$\text{Chls a + b } (\mu g/mL) = 22.12\ (\text{Abs } 652\text{ nm}) + 2.71\ (\text{Abs } 665\text{ nm})$$

4.4.4. Using chlorophyll concentration measurements as a guide, remove and discard some volume from each sample, as necessary, so that each tube contains the same total amount of chlorophyll.

4.5. Re-pellet thylakoid membranes by centrifugation at 5,000 x g for 10 min. Remove and discard the supernatant. Be careful to remove all supernatant without aspirating any of the pellet.

5. Solubilization of Thylakoid Membranes for Loading onto Native Gels

5.1. Thaw an aliquot of TMK 30% glycerol detergent solution (SB) and invert several times to mix. Keep it on ice.

5.2. Dissolve the thylakoid pellet by adding the appropriate volume of SB to give a chlorophyll concentration of 1 mg/mL.

Note: This concentration is a starting point for finding the optimal solubilization conditions, which must be determined empirically. The chlorophyll concentration must be kept the same between samples to allow valid comparisons to be made.

5.3. Pipette up and down repeatedly while being careful to avoid frothing of the sample. Keep on ice to allow thylakoid samples to solubilize.

Note: Solubilize for at least 10 minutes. Solubilization time should be long enough that the difference in solubilization time between samples is minimized.

Example: If 3 minutes are required to solubilize all samples, then approximately 30 minutes should be allowed for solubilization.

5.4. Centrifuge solubilized thylakoids at 10,000 x g at 4 °C to pellet insoluble material.

Note: Solubilized thylakoid samples are stable on ice for hours, but storage at -70 °C should be avoided, as freeze-thaw cycles can result in a loss of megacomplex bands.

6. **Separation of Solubilized Thylakoid Proteins by Native Gel Electrophoresis**

6.1. Load the solubilized thylakoid supernatant prepared in step 5.4 directly onto the native gel prepared earlier. For a 1.5 mm gel, load 15 μL of solubilized thylakoid per well.

6.2. Run the native green gel in essentially the same manner as SDS-PAGE gels using 1x running buffer. Run the gel at 100 V and place the entire gel tank on wet ice for the duration of the run to mitigate resistive heating of the gel.

Note: The gel should require approximately 2 hours for the free pigment at the migration front to reach the bottom of the gel (**Figure 1).**

7. **Excision of Thylakoid Complex Bands from Native Green Gels**

Note: Excising the specific band of interest from the gel is necessary to allow the band to be placed in the beam path and to prevent stray fluorescence from nearby complexes from being collected.

7.1. Remove the gel from the electrophoresis cell and rinse running buffer off of the gel plates with distilled water. Remove the top plate from the gel and rinse the gel with distilled water.

7.2. Keep the gel on the bottom glass plate and place the gel and plate on ice. When not in use, keep the gel in the dark and cover with a plastic wrap to prevent it from drying out.

7.3. With the gel remaining on the glass plate, excise each band of interest when ready for TCSPC analysis. Excise bands cleanly with a sharp scalpel or razor blade and take care that the excised band contains no contaminating band material.

8. **Collection of Room Temperature Steady-State Fluorescence Spectra**

8.1. For each complex that will be analyzed by TCSPC, a room temperature fluorescence spectrum is taken between 600 and 800 nm using a fluorescence spectrometer.

Note: The excitation wavelength used to collect this spectrum must match the wavelength used for TCSPC.

9.. **TCSPC of Green Gel Bands**

Note: Refer to **Figure 2** for a depiction of the TCSPC setup.

9.1 Sandwich the gel slice between two glass microscopy slides. Use masking tape, placed on each end of one of the microscopy slides and folded over several times, to create spacers so that the slides can be held together firmly without compressing the gel slice.

Note: This will create a path for the laser beam to pass between the microscope slides and through the gel slice.

9.2. Add a small amount of water to the gel slice at the edge of the glass slides to create a smooth interface that will reduce signal scattering.

9.3. Clamp the gel/slide sandwich in the beam path so that the beam strikes the gel slice through the open edge of the plates, allowing fluorescence emission to be through the side of the glass slides in which the gel is sandwiched, perpendicular to the beam path.

Note: The excitation wavelength used will depend on the experiment. A wavelength of 435 nm will excite both chlorophyll a and chlorophyll b, while 465 nm will preferentially excite chlorophyll b. In this case, 435 nm was used as the excitation wavelength.

9.4. Collect 10,000 total data points at regular intervals across the fluorescence emission spectrum for each complex. For example, collect data every 10 nm, starting at 680 nm and ending at 750 nm.

Note: Prepare multiple gel bands for each complex to be studied so that fresh sample is available in the event that photobleaching prevents adequate signal collection.

10. **TCSPC (Data Analysis)**

10.1. For a given complex, first normalize the peak height of each decay curve for all wavelengths collected.

Note: This step is not necessary for the construction of DAS but allows for decay curves to be overlaid and compared visually with one another as a first inspection of the data.

10.2. Tail-match each decay curve to the steady state fluorescence spectrum of the complex as described below.

10.2.1. Choose a timepoint after which the decay signal has flattened out, usually after a few nanoseconds.

10.2.2. For each wavelength, normalize the decay curve so that the signal intensity at the selected timepoint is equal to the value of the steady state fluorescence spectrum at that wavelength (*i.e.,* the values of all wavelengths together at the selected timepoint will re-create the steady state fluorescence spectrum).

10.3. Build decay-associate spectra (DAS) from the tail-matched decay curves, as described below.

10.3.1 Using data points from the tail-matched decay curves construct a series of plots graphing fluorescence intensity vs. wavelength at regular time intervals (*e.g.,* every 10 ps). For example, the DAS at 50 ps is constructed by plotting the value of each decay curve at 50 ps vs. wavelength.

10.3.2. Overlay all of the decay-associated spectra to create a waterfall style plot that shows the decay of the fluorescence spectrum over time.

REPRESENTATIVE RESULTS:

Representative results for green gel electrophoresis are presented in **Figure 1**. Lane 1 provides an example of ideal results for green gel electrophoresis of spinach thylakoids, in which a maximum number of clear, sharp green bands are visible. These results are somewhat atypical, in part because not all of the bands seen in lane 1 are normally present in a given sample. Additional sample cleanup, in the form of chloroplast isolation before thylakoid solubilization, and gradient gel electrophoresis (4-7% acrylamide) are also normally necessary to achieve optimal results. Lanes 2 and 3 present more typical results achieved using the protocol detailed here in conjunction with a 5% non-gradient gel. Lanes 4, 5, and 6 provide an example of poor results due to increasing degrees of under-solubilization of the thylakoid sample. Lane 7 provides an example of typical results achieved with *Arabidopsis* thylakoids instead of spinach. Note that for *Arabidopsis* the megacomplex bands at the top of the gel tend to be poorly resolved compared to those in spinach.

A graphic depiction of the TCSPC setup used for collecting data from native green gel bands is shown in **Figure 2**. **Figure 3** shows a typical workflow for beginning analysis after TCSPC data has been collected as described in step 10. When TCSPC data is collected, each curve at a given wavelength represents an arbitrary number of data points, or "counts". When these curves are overlaid with one another, as shown in **Figure 3A,** the curves cannot be compared with one another directly because they are not represented at the same scale and may not all be registered to the same starting time point. The first step in data analysis is therefore to compare each curve to its corresponding instrument response function (IRF). The peak of the IRF for a given curve is set to time t = 0, and the leading edge of the corresponding fluorescence decay curve is set to overlap the leading edge of the IRF, as shown in **Figure 3B**. This will set all curves to the same time register for later analysis.

While it is not necessary for the construction of DAS, normalizing all curves to the same peak height at this point allows a useful comparison between curves to be made as a first analysis of the data. In **Figure 3C**, the same decay curves for LHCII presented in **Figure 3A** are shown after

time registration, as in **Figure 3B**, and peak height normalization. As seen in **Figure 3D**, peak-normalized decay curves from different complexes can then be overlaid with one another at a given wavelength, allowing the differences in behavior between the complexes to be visualized. For example, LHCII has a characteristically long-lived fluorescence that decays slowly, whereas PSI fluorescence is strongly quenched, decaying very rapidly. The Band 5 fluorescence decay curve provides an interesting example of very suggestive data, in part because it is clearly biphasic. The initial fluorescence decay curve for the Band 5 complex follows the same rate as PSI for approximately 500 ps yet decays even more rapidly thereafter. Intriguing results of this kind can be analyzed in further detail by constructing decay-associated spectra (DAS).

In **Figure 4**, representative DAS waterfall plots are shown for LHCII and the Band 5 complex. Construction of DAS first requires that the decay curves for a given complex be tail-matched to the room temperature fluorescence spectrum for the complex, as described in step 11.2. The results of tail-matching decay curves for LHCII are shown in **Figure 4A**. DAS are then constructed from these curves as described in step 11.3. DAS for LHCII were constructed from the decay curves shown in **Figure 4A** and the results are presented in **Figure 4B**. DAS between 0 and 100 ps were omitted for clarity, and only presented every 100 ps thereafter due to the characteristically slow decay for isolated LHCII. The DAS for LHCII is notable for the lack of dynamic features, and the shape of the LHCII fluorescence spectrum remains the same as the signal decays over time. The decay of the fluorescence spectrum is also delayed, requiring 100 ps to reach maximum fluorescence. This suggests, as would be expected for isolated light harvesting protein complexes, that energy is not transferred between energetically distinct pigments within the complex as the fluorescence decays. The exception to this is the shift in the spectrum occurring during the first 100 ps, presumably due to the initial redistribution of excitation energy throughout the complex.

DAS for the Band 5 complex are shown in **Figure 4C**, constructed in a similar fashion to that shown for LHCII. Band 5 provides an instructive contrast to LHCII in several ways. Compared to LHCII, fluorescence from Band 5 decays much more rapidly, reaching maximum intensity in only 30 ps and decaying to less than 20% of initial intensity after 500 ps. The fluorescence spectrum of the Band 5 complex also exhibits a number of interesting dynamics as the emission decays. Comparing the spectra at 0 and 60 ps clearly shows an increase in fluorescence at 720 nm at the expense of fluorescence at 680 and 710 nm. Thereafter, the peak at 680 nm shifts towards 690 nm and broadens, while the peak at 720 nm shifts back toward 710 nm (*e.g.*, compare 60 ps to 500 ps). Data of this type helps rule out the presence of unconnected LHCII antenna proteins while providing evidence for energy transfer from LHCII to the PSI antenna and eventually the PSI core.

These dynamics also suggest that there are likely to be unresolved peaks around 680 nm, 690 nm, 710 nm, and 720 nm. This data therefore provides an example where spectral features could be better resolved by collecting TCSPC data at closer intervals (*e.g.* every 5 nm rather than every 10 nm). Even in the absence of higher spectral resolution, however, the DAS for the Band 5 complex are an example of evidence for energy transfer between multiple fluorescing species within a complex. There also appears to be a rapidly decaying peak above 740 nm, suggesting

that data should also be collected over a broader spectrum to include further spectral features.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative green gel results. Green gel bands subjected to TCSPC analysis are labeled PSI-LHCII (photosystem I LHCII megacomplexes), PSI (photosystem I LHCI), Band 5 (PSI-LHCII complex), PSII-LHCII (photosystem II and associated light-harvesting complex II), PSII (photosystem II core complex), and LHCII (light-harvesting complex II). Lane 1 shows an ideal banding pattern resulting from chloroplast isolation before solubilization and green gel electrophoresis on a 4-7% acrylamide gradient gel. Lanes 2 and 3 show average results from simple thylakoid isolation and electrophoresis on a non-gradient 5% acrylamide gel. Lanes 4-6 show increasing severity of under-solubilization. Lane 7 shows representative results for Arabidopsis using the simple protocol.

Figure 2: Schematic of time-correlated single photon counting instrument. Light source is a passively mode-locked $NdYVO_4$ laser that pumps a cavity dumped dye laser. 5-ps pulses at variable repetition rates and wavelengths are characterized using an autocorrelator. The pulses are divided, with one portion going to a reference photodiode and the rest exciting the sample. Sample emission is collected using a microscope objective, and polarized components are detected using two detection channels. Sample emission is time resolved using the detection electronics indicated, where CFD = constant fraction discriminator, TAC = time-to-amplitude converter, and MCA = multi-channel analyzer. Time resolution is determined from the instrument response function (ca. 40 ps).

Figure 3: Representative raw TCSPC data and normalized fluorescence decay curves. (A) Overlay of raw TCSPC data for LHCII. TCSPC data was collected every 10 nm between 670 nm and 740 nm (B) A representative curve showing time registration of the fluorescence data to the instrument response function (IRF). (C) The LHCII data from (A) normalized to a maximum peak height of 1 after all curves were registered to their respective IRFs. (D) Overlay of fluorescence decay curves at 680 nm for PSI, PSII, PSII-LHCII, LHCII, and Band 5. All decay curves were normalized to a maximum peak height of 1.

Figure 4: Construction of waterfall-style DAS. (A) Tail matching of the decay curves for LHCII in preparation for construction of DAS plots. (B) DAS plots for LHCII for time t = 0, for every 100 ps from 100 to 500 ps, and at 1000 ps. (C) DAS plots for Band 5 every 30 ps from t = 0 to 210 ps and every 100 ps thereafter to 500 ps. For both (B) and (C), time = 0 is defined by the IRF, which is 40 ps.

DISCUSSION:

A successful thylakoid solubilization and native gel run will result in the resolution of multiple distinct visible green bands on the gel without significant distortion or smearing of the bands. Overloading the gel, a high detergent concentration, an incorrect sample pH, undissolved material, running the gel too rapidly or at too high a temperature, and an improperly poured gel are all factors that may contribute to poorly resolved thylakoid complexes. While optimizing the conditions of the gel itself (*e.g.*, acrylamide gradient concentrations), it can help to maximize the

resolution of bands of interest. It is our experience that solubilization conditions and the biology of the sample material itself are the most important factors contributing to the quality and quantity of thylakoid complexes resolved on native green gels. It is important to remember that not all complexes will be present under all biological conditions.

Native green gels are poured in essentially the same manner as normal SDS-PAGE mini gels. Gels can be poured in the morning and are ready to use later the same day, or they can be stored at 4 °C for several days with the comb in the gel and no significant changes in performance. Standard, readily available 39:1 C acrylamide solutions can be used to pour both the stacking and resolving gels. So-called “large pore” gels can be made using higher acrylamide:bis-acrylamide ratios (*e.g.*, 100:1 C) in order to increase separation of megacomplexes at the top of the gels, but we find that this also tends to result in less sharply resolved bands. In our experience, the highest band resolution (with the greatest number of bands separated) is achieved at the lower C ratio and with a linear acrylamide concentration gradient of 5-7%. However, if a gradient former is not available, very satisfactory band separation and resolution can be achieved with a resolving gel concentration of around 5% acrylamide. It is important that electrophoresis be carried out at relatively low voltage to reduce heating of the gel, which could denature the complexes, and to allow native complexes to separate from one another with high resolution.

The method for thylakoid isolation given here is rapid but relatively "dirty" *(i.e.,* it does not attempt to separate chloroplasts from other organelles or thylakoid membranes from other cell membranes). This is sufficient for the purposes of visualizing thylakoid complexes and subsequent fluorescence-based measurements, since only chlorophyll-containing proteins are visualized. It should be noted that for other downstream applications (*e.g.*, second dimension SDS-PAGE and protein detection), non-thylakoid proteins will be present. It should also be noted that the best resolution is achieved when chloroplasts are isolated before solubilization, presumably due to the removal of insoluble materials before solubilization. When following the method described here, other homogenization methods such as a blender may be used, but a glass Dounce homogenizer is rapid and effective and allows all homogenized leaf material to be retained quantitatively. The ratio of buffer-to-leaf material does not appear to be important, to our knowledge. Approximately 2 mL of buffer for one half of a baby spinach leaf is a convenient starting point but may be adjusted based on experimental needs.

The appropriate ratio of solubilization buffer to chlorophyll is crucial for optimization of green gel performance and should be determined by the experimenter for a given plant species or experimental setup. Proper solubilization will result in a clear, deep emerald-green supernatant above a white starch pellet. A layer of green material on top of the white pellet indicates that the thylakoids have been under-solubilized. Under-solubilization must be avoided, as it will result in poor migration on the gel, including smearing of the bands, and will not provide an accurate banding pattern. The thylakoid pellets produced by this method should be easy to resuspend and solubilize. In case of compact pellets which cannot be resuspended quickly and homogeneously an alternative method is recommended. Samples can first be resuspended in half the volume of TMK-glycerol buffer, to which is then added an additional half-volume of TMK-glycerol buffer containing a 2X concentration of detergents.

Over-solubilization should also be avoided, as it may result in the loss of density of some megacomplex bands. Additionally, it is not possible to make up for over-solubilization by loading a larger volume of sample onto the gel - we have found that loading more than 15 μL of the sample per well on a 1.5 mm gel reduces the quality of the separation; although, doing so may be desirable in order to visualize complexes with low abundance. Conversely, loading less than 15 μL can improve band resolution and reduce smearing but will reduce the visibility of some low-density bands. In general, both increased protein concentration and increased detergent concentration contribute to band distortion and gel smearing.

For time-correlated single photon counting (TCSPC) analysis of the green gel complexes, the excitation and emission wavelengths must be chosen by the experimenter at their discretion. For our purposes, the chosen excitation wavelength was 435 nm, which will excite both chlorophyll a and chlorophyll b. Different wavelengths can, however, be used to excite specific chlorophylls or other pigments more selectively (*e.g.,* an excitation wavelength around 465 nm will preferentially excite chlorophyll b). Similarly, a fluorescence emission spectrum covering a region from 680 to 740 nm was selected based on the reported emission spectra for LHCII, PSI, and PSII. Therefore, this region covers all the relevant spectral features of interest for our purposes. The wavelength intervals at which data are collected are also up to the discretion of the experimenter. Shorter intervals, such as every 5 nm instead of every 10 nm, will make it possible to construct a more detailed decay-associated spectrum, but this requires more time and may not be necessary. Conversely, longer intervals may fail to resolve relevant spectral details.

Simply overlaying normalized fluorescence decay curves from different complexes can provide revealing information about those complexes. Fluorescence from LHCII, for example, is characteristically long-lived, while fluorescence from PSI decays much more rapidly. Care should be taken at this point to examine data against the instrument response function (IRF) to ensure that the observed decay kinetics are temporally resolved from the response time of the instrument.

Constructing decay-associated spectra (DAS) from the TCSPC data creates a much more detailed picture of energy transfer between chromophores within a complex than what is possible from simply looking at isolated decay curves. When constructing DAS, tail matching of the TCSPC decay curves to the steady state fluorescence spectrum is necessary because the intensity of signal collected by TCSPC is arbitrary. At long time points such as 5,000 ps, however, the fluorescence intensity at a given wavelength (the “tail” of the decay) is the same as the steady state fluorescence intensity for that wavelength. The steady-state fluorescence spectrum can therefore be used to normalize all of the TCSPC decays so that their tails are equivalent to the steady state spectrum. This gives the true relative intensity of fluorescence signal for each decay curve. The entire series of DAS, when overlaid together in waterfall plot style, provides a graphic depiction of the decay of the fluorescence spectrum over time. If the plots are carried out to long enough time points (to the tails of the decay curves) the waterfall plot will decay all the way to the steady-state fluorescence spectrum. The earliest time point, on the other hand, is determined by the response function of the TCSPC instrument.

ACKNOWLEDGMENTS:

Funding and support were provided by the Department of Chemistry at Michigan State University.

DISCLOSURES:

The authors declare no conflicts of interest.

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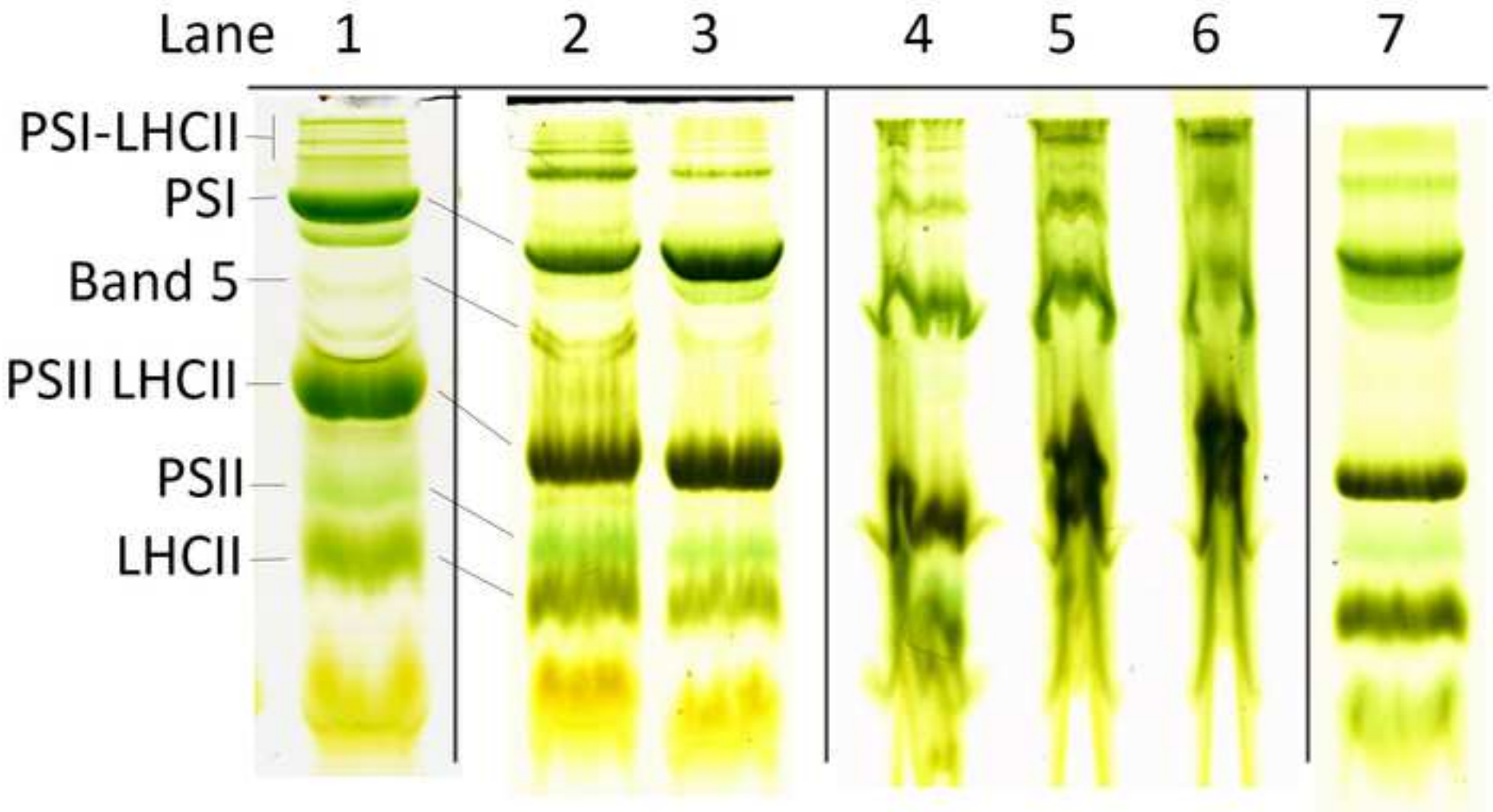

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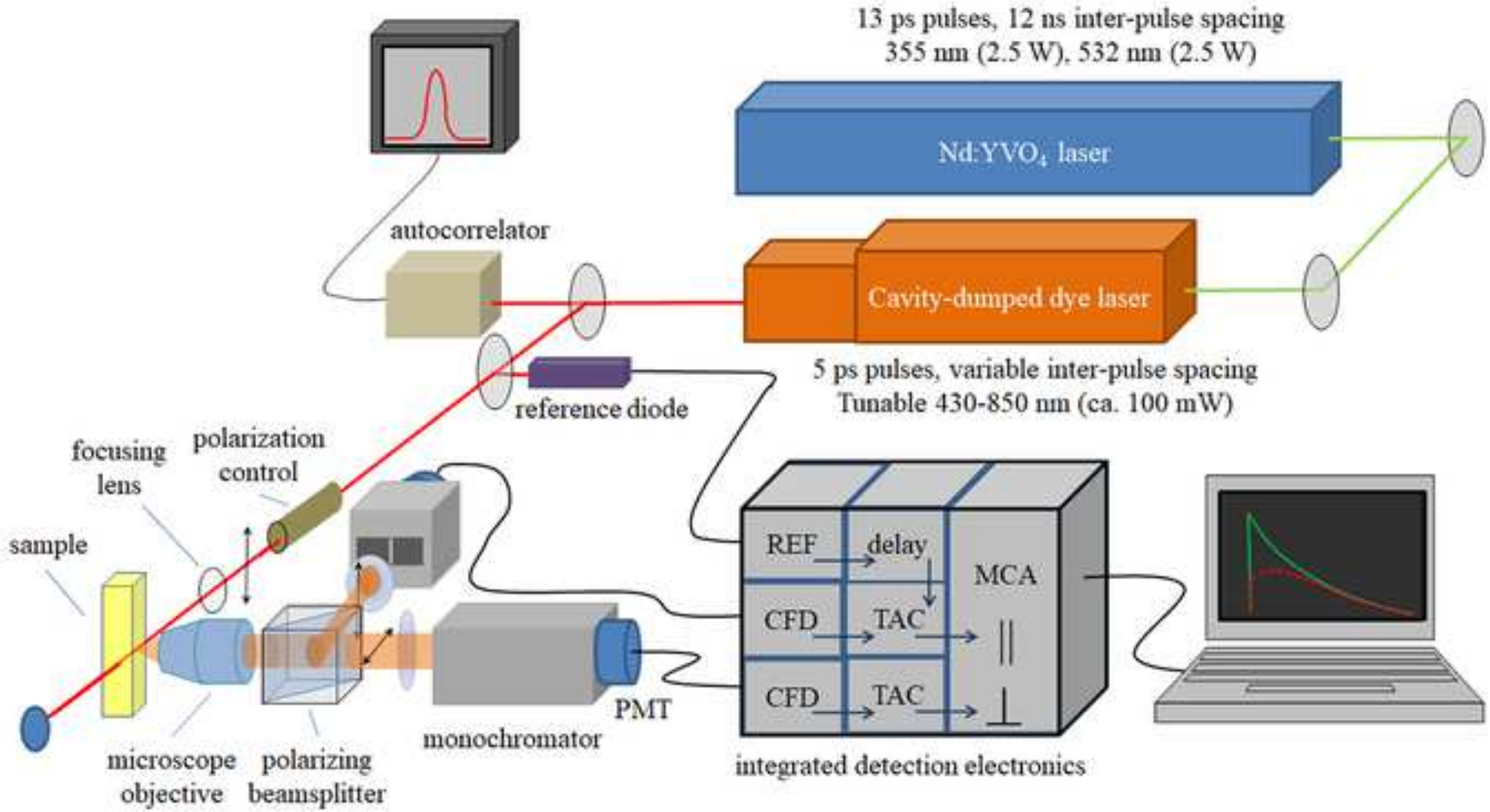

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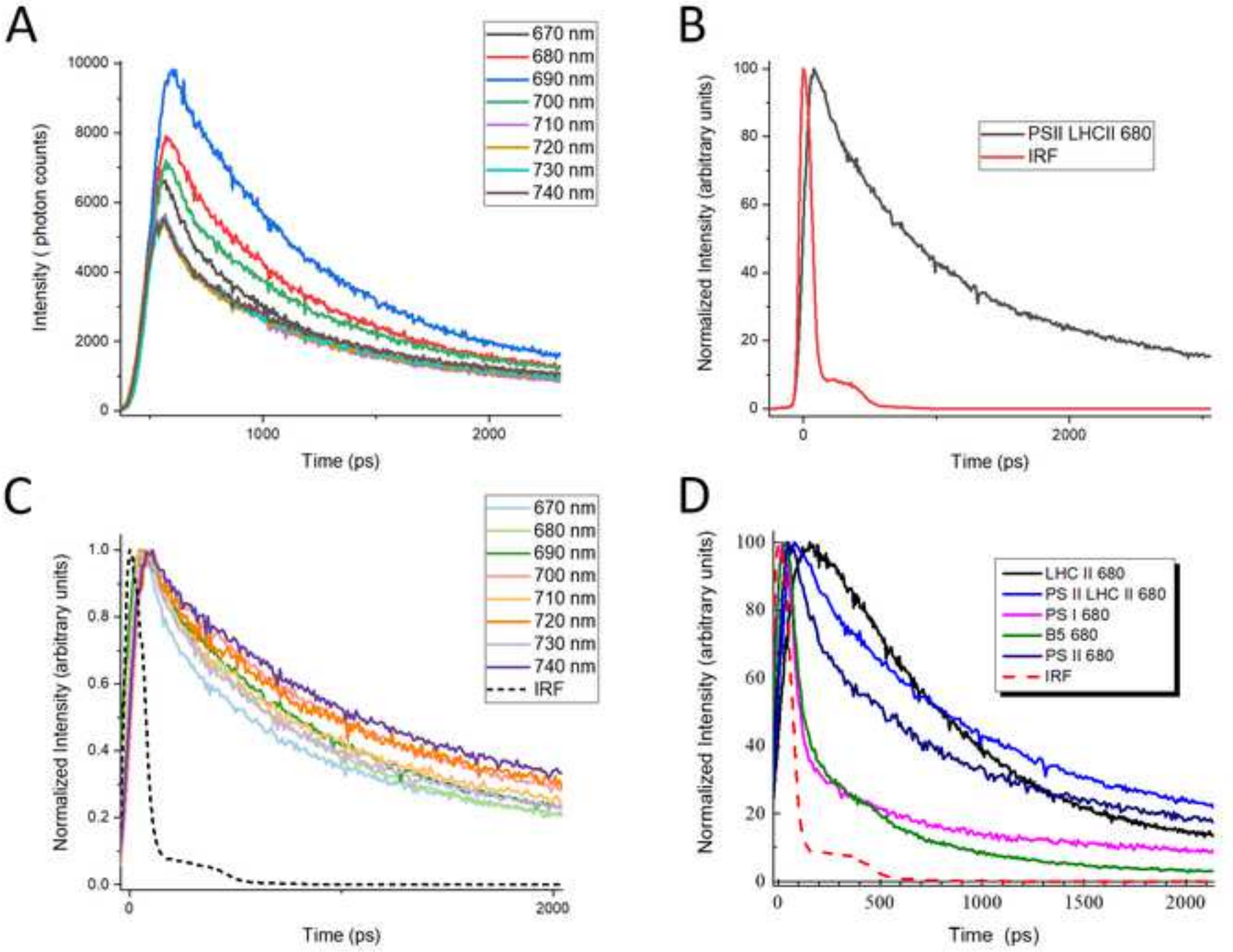

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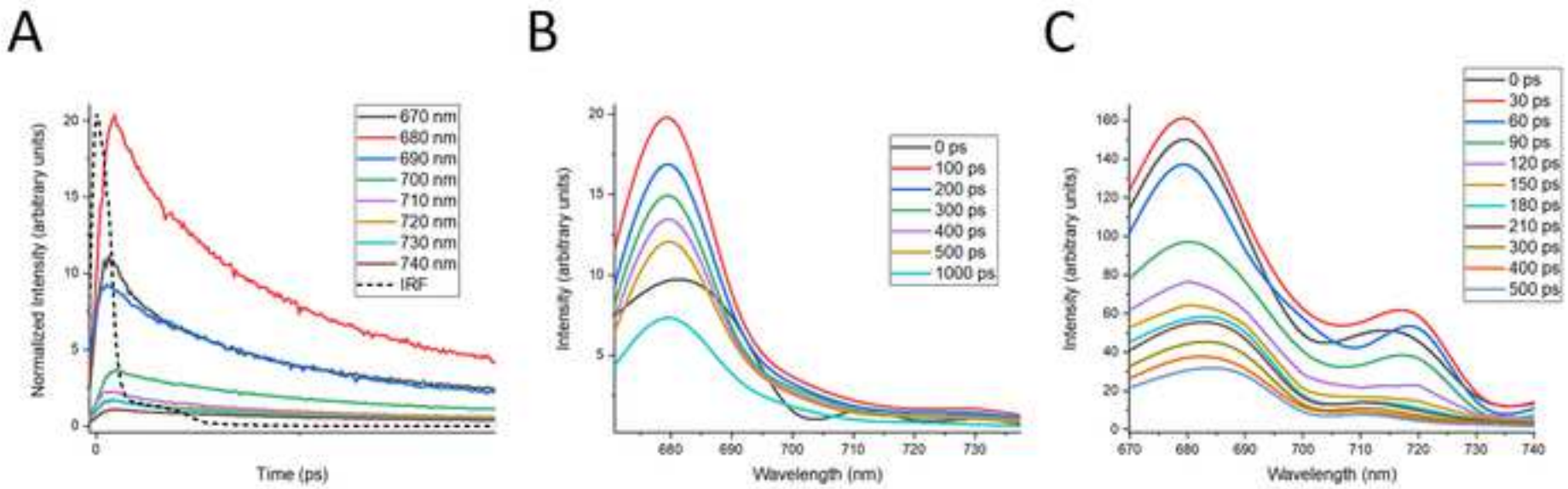

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Glycine	Sigma	G8898	
Tris base	Sigma	#648310	
SDS	Sigma	L3771	
Decyl Maltoside	Sigma	D7658	n-decyl beta d maltopyranoside, not dodecyl maltoside or alpha decyl
Octyl Glucoside	Sigma	O8001	
Acrylamide	BioRad	161-0148	37.5/1 C 40% solution
TEMED	BioRad	161-0800	
Ammonium Persulfate	BioRad	161-0700	
Magnesium Chloride	Sigma	M2670	
Potassium Chloride	Sigma	P9333	

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Reviewer #1:
Manuscript Summary:
In this manuscript, Schwarz and Blanchard develop a method to make possible the detailed description of energy transfer between spectral components in a photosynthetic complex via native green gel electrophoresis followed by band characterization using a time correlated single photon counting. Overall, the manuscript is the scope of JoVE but the scope of this work itself is very limited.

Major Concerns:
The scope of this work itself is very limited. Please add some additional information to expand the scope of this work itself, for example, who might be interested in using this method.

Please see lines 87-94

Minor Concerns:
1. Lines 217 and 218: Please correct the sentence. SDS has to be absent in the whole process of native green gels.

We refer the reviewer to Allen and Staehelin for a discussion of this topic

2. Please update the pictures of Figures 2 and 3, and their quality is very bad.

This has been done.

Reviewer #2:

In the abstract, the description for other methods (cumbersome for EM and low resolution for sucrose gradient centrifugation) is totally wrong and should be avoided in this type of journal.

We realize that these descriptions can be always be considered subjective and have amended the text to avoid negative comparisons with other useful techniques

Lines 53-54
NPQ is collective term for qE (energy-dependent), qT (state transition-dependent), and ql (photoinhibition). When the authors name NPQ, that includes all three. So NPQ and state transition do not stand equally. PSII repair cycle is not equivalent to ql. The authors should revise all that apply the misuse of NPQ for qE.

In the interest of brevity the descriptions of photoprotective phenomena provided in the introduction sacrificed a degree of accuracy. The text has been amended to reflect this.

Lines 62
The paper for TAP38 should be included, not just PPH1 phosphatase.

Line 65
After Larsson et al. (1983), numerous updated studies have been published, and I think that Larsson et al. includes only limited knowledge about state transitions to cite.

As the reviewer states, it is certainly true that much has been learned since the publication by Larsson et al. in 1983. The preceding sentences, for instance, both mention and cite the finding of the kinases and phosphatases involved in the phosphorylation and dephosphorylation, respectively, of LHCII. Larsson is specifically cited here as the historical reference to the original demonstration that state transitions reflect the physical movement of LHCII due to phosphorylation. If the reviewer would like to recommend other refereces for this purpose we will include them along with more recent references that have been added.

Lines 67-68

The statement here is totally wrong, there are many other studies using various different techniques to understand the detailed mechanisms. I'm afraid that the authors do not appreciate the knowledge that has been stored in the literature by significant number of researchers for a long time.

The reviewer has perhaps mistaken the rhetorical purpose of this statement. We naturally agree that much more is known about the various aspects of NPQ, both their biophysical manifestations as well as the biochemical mechanisms from which they arise, than that they are known "simply as a loss of chlorophyll fluorescence that is not rapidly reversible". We have amended the text in an attempt to make our meaning more obvious, which is to say that the reason for the very name "non-photochemical quenching" attached to these phenomena arises from their original description based on spectroscopic observations.

Lines 75-77

The statement here is again totally wrong. Many biochemical researchers in this field have tried to develop different techniques that are suitable for different types of proteins and protein super/complexes and appropriate for different purposes. Sucrose gradient centrifugation is mainly used to keep protein samples intact, while separating a variety of protein super/complexes by using different detergents and/or centrifugal forces. Column chromatography can be used for variety of separation (size-exclusion, ion-exchange, affinity purification), and there are many proteins which only separable by column chromatography. Native-PAGE is more handy, and nowadays precast gels are available, so it is very easy to access to many labs. This technique does not require a large amount of sample, either. Each technique has its own pros and cons, and many researchers choose an appropriate technique for a problem to solve. Therefore, it is not the resolution to separate proteins. Also, I must say that it is not true that Native-PAGE has a better ability to separate proteins compared to other techniques, which is shown in Figure 1. There are many proteins that Native-PAGE cannot resolve.

The reviewer makes a number of statements concerning the use of a variety of techniques, none of which the authors consider to be in dispute. It is fully agreed that there are various techniques which may be used for various purposed to achieve various ends, and further that some of these techniques may be more or less well suited to achieve such ends or more or less preferred by various experimenters. We also do not contend that Native PAGE is superior to other techniques in all cases. We do not, for instance, make the broad claim, as suggested by the reviewer, that Native PAGE can separate all proteins better than other techniques or that there are not proteins that cannot be separated by Native PAGE. Such claims would be preposterous.

Rather, this section is intended to provide some historical context for green gel electrophoresis, based on facts which are reflected in the publication record. The groups from which green gel electrophoresis emerged appear to have developed their methodologies based on previous efforts to separate thylakoid complexes using chromatography and sucrose gradients. The application of detergent and buffer systems developed in these efforts to gel electrophoresis led to the resolution of a large number of discrete thylakoid complexes. The paper by Allen and Staehelin in particular is specifically devoted to the development of a green gel system capable of resolving a large number of physiological thylakoid complexes. To our knowledge there had not yet been, nor indeed are there now, methods for resolving upwards of sixteen native thylakoid complexes simultaneously by other techniques. We have nevertheless removed the pejorative implication regarding such techniques from the text.

Line 81

Native-PAGE (green, clear, or blue) is the easiest technique than sucrose gradient centrifugation, column chromatography, or others. And the statement here is wrong because any techniques require a significant investment of time and talent without prior experience.

The reviewer states that Native PAGE "is the easiest technique". The reviewer will then presumably allow that some techniques can be easier to learn or easier to implement than others, therefore not requiring as much time and talent without prior experience. We presume that the purpose of a methods journal like JoVE is, at least in part, to provide guidance for implementing potentially troublesome techniques without prior experience or guidance by those familiar with them. Whether or not Native PAGE is easier than other techniques, it appears true that such guidance would be of value.

Line 151
The description of gel plate is missing, such as plate size or thickness of the gel. The comb size should be mentioned as well because unlike regular SDS-PAGE, Native-PAGE requires larger sample volume.

added

Lines 196-201
This quick reading at 663 nm method should not be recommended as not only it is not suitable for samples with different Chl a/b ratio but also it is not the total amount of chlorophyll (rough chlorophyll a without compensate inclusion of chlorophyll b).

Changed to clarify

Line 208
Adding SB to thylakoid pellets is not recommended because pipetting is required to resuspend the pellet, and pipetting may produce bubbles, which interfere solubilization differently. Pipetting each sample takes time, which causes the delay of the time of solubilization per sample, which is not a properly controlled experiment. Many researchers add SB to resuspended samples, sometime add SB dropwisely to the sample that is slowly mixed. This way detergents will interact membranes and proteins more evenly and reproducibly. No one knows how pipetting affect the solubility of the samples, but adding SB to the pellet is not a widely accepted way for sure.

The reviewer makes a valuable point; resuspension prior to solubilization is good technique, especially when pellets may be difficult to resuspend. We agree that compact thylakoid pellets, such as those produced by Arabidopsis, especially when pelleted at higher G forces, can be difficult to resuspend. Inexperienced pipetting technique can then easily lead to frothing and uneven solubilization. Spinach thylakoids that are pelleted at lower G forces, as described here, yield a relatively fluffy pellet that is quite easy to resuspend, and homogeneous solubilization is accomplished very quickly. The reviewer states that "No one knows how pipetting affect (sic) the solubility of the samples". We do know this, having compared methods. Our experience has revealed no difference, as long as pipetting is done carefully. That being said, resuspension prior to solubilization is a useful technique and the text has been amended to reflect this.

Line 217
It is interesting to see that the SDS-PAGE running buffer is used for Native-PAGE.

We agree with the reviewer that this is interesting. For a discussion of this specific topic we refer the reviewer to Allen and Staehelin.

Line 220
How to excise the gel is poorly described although this would be the main purpose of this study. In which solution is gel band excised? Which type of blade? Cut it on the glass? How do you store the excised bands until TCSPC and in which solution?

The text has been amended to include these details

Line 225
No description about getting IRF is mentioned, which I imagine should be specific for this type of samples.

Line 277
What is the rationale to use peak heights for normalization? In DAS analysis, one can normalize by a certain wavelength at a certain lifetime.

The reviewer is correct that peak normalization is not necessary for construction of DAS. However, as described in the text, it is useful and informative, especially when one is new to TCSPC or when working with new complexes, to visually compare decay curves as a first inspection of the data.

Lines 354-355
It is not recommended to use such dirty prepped samples and provide such data out there. To obtain higher resolution, it is always necessary to isolate clean purified samples

While we agree with the reviewer in spirit that high standards are necessary to achieve reliable data and that best practices should always be followed, it is also true that adherence to standards and practices that serve no empirically necessary purpose only serves to impede progress and obscure good practice. The terms 'clean' and 'purified' are necessarily relative, and the degree of cleanliness and purity required can only be defined as a function of the results they produce. We believe that our results testify for themselves, and that individual experimenters, as well as the scientific community in general, can benefit from an understanding of the types of results that can be expected from different types of preparations. This seems especially appropriate in the context of a methods paper.

Lines 398-400
This is right to normalize the DAS data, but as mentioned above, the presented here is normalized by the peak height. Why is that?

See above

Figure 1
In Protocol section, only a constant (non-gradient) gel is described, whereas in Figure 1 the result using 4-7% gradient gel is shown. It is clear that a constant gel is not suitable for readers to use. But yet the authors chose not to describe how to make a gradient gel. Also, I must say that even with gradient gel the separation is not very well despite that the authors claim Native-PAGE has higher ability to separate protein complexes than other techniques.

Figure 1 shows results for different kinds of outcomes, ranging from optimal to poor, as desired by JoVE. The procedure provided here can incorporate a gradient gel or not. The method for pouring a gradient gel is not described here in part because such descriptions and demonstrations can easily be found elsewhere, and because they may vary depending on the gradient former used.

We cannot properly evaluate the reviewer's opinion that non-gradient gels are not suitable for use, as the reviewer does not describe criteria by which the suitability of a given gel system may be judged. Nevertheless, we would argue that a gel system is suitable if it is capable of separating and visualizing the complexes of interest. Since the non-gradient gel resolves nearly as many bands as a gradient system, it is arguably a sufficient system in many cases. To the extent that the resolution of a non-gradient gel is worse than that of a gradient gel, i.e. that bands can not be sufficiently separated for subsequent TCSPC analysis, the remedy for this problem will depend on the experimenter's discretion: resolution of the band of interest may be improved by using a gradient gel, by underloading the gel, by increasing separation time, or possibly by further sample cleanup. The choice will likely depend on the position and density of the band, as well as the experimenter's own disposition. This paper is intended to provide the potential user of these methodologies with information that will be useful in evaluating their results and the options available to them.

With regards to resolution, we are not aware of any evidence in the literature which demonstrates sucrose gradient centrifugation of thylakoid complexes with capabilities similar to, let alone surpassing, Native PAGE, either in terms of resolving power or number of bands resolved, and we would be happy to learn of any such technique. In terms of resolving power or theoretical maximal resolution, the minimum band thickness in sucrose gradients is relatively much larger than that seen in Native PAGE. In practical terms this is readily apparent when looking at megacomplexes on Native PAGE, which can be resolved into a series of very fine bands, as in Figure 1, whereas these bands are not at all resolved on sucrose gradients (e.g. Xu et. al. 2015). In terms of the number of complexes resolved, it has been typical for sucrose gradients to resolve between six and eight bands. To our knowledge there are no examples of resolution of sixteen or more thylakoid complexes by sucrose gradient centrifugation, although we would be grateful to be informed otherwise.

Reviewer #3:
Manuscript Summary:
Schwarz and Blanchard propose a step by step description of a method for the characterization of native pigmented thylakoid complexes coupling a biochemical separation with a biophysical analysis. The rationale for the method is valid, because it responds to the need for ever better methods for native green band identification.

Concerns:
The title is somewhat misleading, because it emerges that the focus is not just describing a green PAGE protocol, but proposing a method faster than other commonly used - and an important aspect is that the resolution quality can be lower indeed. Therefore, a researcher willing to use this method should carefully consider pros and cons.

The text has been amended to more accurately reflect the pros and cons of the method presented and the reasons for its potential use

In the table, the authors list reagents for electrophoresis, but not for thylakoid separation. Moreover, the TCSPC method needs equipment not described in this ms. One of the authors used this method in a very recent paper (Schwarz et al., 2018, Photosynthesis Res. 136, 107-124), providing some details about equipment, which seems in-house made. I think it is vital for this method ms to thoroughly describe this equipment, possibly including a diagram illustrating it.

A diagram and description of the instrument have been included

There are many other points needing attention by the authors.

Introduction:
In some instances the text is too vague. In particular, the light reactions of photosynthesis are called "light-induced reactions" in line 46, which is imprecise (also Rubisco' reaction is light-induced). The subsequent sentence on photooxidative damage to the "components" is too generic when it comes to be referred to their "routine occurrence", in fact the relevance/reversibility of the damage depends on the target - PSI repair after damage is very energy-demanding. In line 53-55, the authors confound NPQ components and related processes, especially when they attribute the qI component of NPQ to the PSII repair cycle, while qI is simply a sustained PSII photoinhibition, not necessarily reflecting a down-regulated repair (see many Demmig-Adams' papers). Similarly, in lines 65-69 the link between PSII repair mechanism and fluorescence is too simplistic and overlooks the recognized outstanding importance of chlorophyll fluorescence for the physiological description of photosynthesis-related phenomena.

In Introduction, the authors do not mention the routine elective analysis for the compositional characterization of native complexes, i.e. second dimension SDS PAGE. A recent paper by Eva-Mari Aro's group further extends the potential of such biochemical characterization and the authors are suggested to acknowledge it (Rantala et al., 2017, Plant J, 92, 951-962).

The introduction has been modified to address these concerns and provide more specific descriptions of the phenomena described

Protocol:
There are many different protocols for thylakoid isolation and solubilisation, they are more or less efficient and reproducible. The method here proposed in detail essentially is that used by Schwarz et al. (2018) in their already mentioned (but not referenced!) recent paper. The goodness of a method is measured on the results it allows and objectively Schwarz et al. showed very good biochemistry in their paper. As JoVE asks for evidencing possible shortcomings in the protocol, I'll drive the authors' attention to the following aspects, using their list numbering.

2.1 Different from other common thylakoid isolation buffers, TMK does not contain any additional neutral compound for osmolarity correction (sucrose, sorbitol). Doesn't this omission impact on thylakoid quality?

The reviewer makes a good observation and an interesting point. It is expected that the thylakoids isolated by this method would not be recommended for use in functional assays, although we do have evidence demonstrating reasonably high rates of electron transport capability. It appears that an osmoticum is often included in thylakoid isolation protocols adapted from chloroplast isolation protocols, and that thylakoids are eventually resuspended without an osmoticum in any event. Homogenizing thylakoids directly into TMK does not seem to have any effect on thylakoid complexes in our experience

The authors suggest that this method is adequate for thylakoid regulatory processes, including state transitions, but in the TMK they omit NaF as a phosphatase inhibitor, so the method cannot give reliable/repeatable results on the state-transition complex, whose assembly depends on phosphorylated LHCII trimers (see lines 61-62). In spite of this being not taken into account in Schwarz et al. (2018), actually also the PSI-LHCII megacomplexes are functionally regulated by reversible phosphorylations (see e.g. Grieco et al. 2015 BBA, 1847, 607-619).

The reviewer makes a useful point and we have amended the text to make a note of the recommended use of inhibitors, at the experimenter's discretion, depending on the processes under study and the design of the experiment. As the reviewer points out, phosphorylation does play a role in the formation of PSI-LHCII megacomplexes. Clearly, the dynamics of such complexes can be reliably visualized even in the absence of inhibitors, likely due in part to the fact that reorganization of complexes is slower than the enzymatic events that may trigger them.

Another non-negligible aspect is the opportunity to amend TMK with some protease inhibitors.
As regards TMK preservation, can TMK be stored at -20° C for longer times?

As above, we have amended the text to note the potential addition of fresh protease inhibitors, at the experimenter's discretion. There does not appear to be any limitation to the storage of TMK at -20 C

3.1 I cant' find what electrophoretic cell was used (or should be used) - I suppose a minicell.

This has been added

3.1.1 The 40% acrylamide should be better specified here with regard to the acrylamide/bis mixture used.

This has been added

3.3.1 All of these steps are the same as in 3.2.1, in particular 50 uL APS and 10 uL TEMED?

This has been added

4.1 Not all labs have the same accuracy during thylakoid isolation and this affects the quality of preparations. Two fundamental aspects not mentioned here are conditions of temperature and ambient light. Under best conditions, thylakoids are isolated on ice using precooled homogenizer or mortar; light must be extremely dim, never direct, preferably green. It is unclear whether ALL of the steps for thylakoid isolation were performed under such conditions.

The reviewer makes a good point. The text has been modified to indicate that all steps should be performed on ice using prechilled equipment. While strong lighting during thylakoid isolation is surely to be avoided, the importance of performing thylakoid isolation in darkness/ non-actinic light vs dim lab lighting conditions would seem to depend on the biological processes under investigation, i.e. plants treated with high light vs plants treated with darkness. In our experience quick sample processing on ice provides robust results, whereas variability tends to arise from biological heterogeneity and unexpectedly complex dynamics that give the appearance of poor reproducibility.

4.2.1 The use of kimwipe can be a smart choice. How does it compare with miracloth filtering?

Thank you. This substitution was made because no difference in results were noticed. Miracloth does not clog as easily.

4.4.3 The spectrophotometric reading at 663 can be a lab routine, but I fear it would not be acceptable for many journals. In particular, consider that in plants the LTRs change the Chl a/b ratio, so a single reading at the absorption maximum of Chl a - disregarding Chl b - can result in uneven gel loadings. Personally I would not use this approximation.

The reviewer of course makes a good point. This heuristic is useful as a first approximation in finding the appropriate solubilization range, as we find it tends to be, for reasons that are not clear, very accurate. Nevertheless, total chlorophyll must always be taken into account and normalized across samples for an experiment. We have made a point to note this in the text and have omitted this procedure for the sake of clarity.

5.2 This is empiric, what's the reason for multiplying the A663 by 200?

This is an empirical

5.2 (duplicated) "at least 10 min". What does it mean? How long can solubilisation last?

Whe have amended the text to address this more fully. Samples can remain on ice in solubilization buffer for several hours, i.e. samples loaded onto a gel in the morning vs those loaded late in the afternoon after sitting on ice give identical banding patterns. This is not true of samples left on the benchtop at room temperature or of samples stored on ice overnight.

5.3 For how many mins? Moreover, I would not freeze thylakoids to be analysed for native complexes. Even one single cycle of freezing-thawing can affect results.

The reviewer makes a reasonable point. We have amended the text to reflect this recommendation

6.2 Please specify the running buffer used. Many labs add SDS to running buffers, but this must not be the case for native gels. Specify at least approximately the time of run.

It is indeed the case that the running buffer contains a low concentration of SDS, as described in Schwarz et al. and in Allen and Staehelin. We direct the reviewer to Allen and Staehelin for their discussion of the use of SDS in native gel electrophoresis. As has been noted by others, this buffer/detergent system preferentially destabilizes larger PSII complexes, and we have amended the text to note this.

7.1 Instruction for TCSPS requires explanations about the equipment.

This has been added

7.3 No info is given about excitation wavelength.

This has been added to the text

Representative results:
I have some problems with this section. First of all, there is a description of "typical" and "atypical" results. It took some time to understand that lane in Fig. 1 was NOT obtained with the described method, but with a variant in a gradient gel. In this sense is it "atypical", i.e., the authors are warning us that we shouldn't expect to see this good resolution? That the best we can achieve is that in lane 2 and 3 - i.e. not the best but one can be content anyway? A certain degree of smearing is expected in the typical case?

The reviewer is essentially correct. JoVE specifically asks for a range of results to be presented so that the reader will have an understanding of what can be achieved vs what might go wrong and what options are available to troubleshoot or improve results. The results presented in lanes 2 and 3 are not necessarily the "best" that could be achieved with the given method (better resolution and less smearing can be achieved simply by loading less sample, for instance), but are supplied as an example of "middle of the road" results. Lane 1 does represent the best results that we have had with this system and, while not exactly atypical, are likely better than what a beginner will achieve without additional optimization and/or further sample processing.

Moreover, it is unclear whether the "undersolubilization" of lanes 4-6 was wanted for demonstration (how?) or it's just obtained by chance.

These were obtained by deliberate undersolubilization for the purposes outlined above, and are rather typical of this problem.

I previously read and appreciated the paper by Schwarz et al. (2018) and it is interesting to notice that band attribution has changed in this ms, so there should be something wrong somewhere. As regards lane 1 in Fig. 1, high molecular weight bands are the same as in Fig. 1 in Schwarz et al.; problems arise from lighter bands.
Band 5 here is PSI-LHCII state-transition complex (or what remains of it, having NaF been omitted form isolation buffer), there it was PSII supercomplexes. In both cases, I see inconsistencies, because PSI-LHCII would be lighter than PSI or the PSII supercomplexes would be lighter than PSI. PSI and PSII dimer have similar molecular mass (and indeed co-migrate in BN and clear native gels); if one adds LHCII trimers to them, they generate heavier complexes.

This is an astute observation. We will note first that Band 5 is not necessarily the PSI-LHCII state transition complex, and the fact that it is not very abundant as presented here does not reflect the omission of NaF from the buffer system. Band 5 is a complex that we have not previously characterized which is present/induced under specific physiological conditions which we are still investigating. The fact that band 5 migrates in the region that it does is part of the reason its apparent identity as a PSI-LHCII complex is surprising.

Subsequent large band here is PSII-LHCII supercomplexes (much lighter than PSI??), there it was co-

migrating PSII monomers and LHCII assembly (possibly the authors meant LHCII trimers). Subsequent band here is PSII, there it was PSII inner antenna subunits (i.e. not assembled anymore).

Again, this is an astute observation and a good point. For the sake of continuity we have amended the designation to PSII LHCII rather than PSII-LHCII. There are a few reasons that the designation of this band as separate PSII and LHCII is problematic, although this work is not intended to specifically address or resolve this question. One is that the "LHCII" band can be further resolved into monomers and trimers, and so we know that the LHCII contained in the PSII/LHCII band is not simply LHC trimer. The PSII/LHCII band also does not behave spectroscopically like it contains free LHC, but does appear to be a PSII/LHCII complex, as supported by the data included here. The potential to resolve issues like these, which cannot be accomplished solely on the basis of 2D-SDS-PAGE and position on a gel, is the justification for this paper.

So what? A crucial information to provide is which interactions are preserved and which are disrupted by the detergent system decylmaltoside-octylglucoside.

We agree with the reviewer, but such an investigation, as is in part provided by Rantala et. al, as the reviewer points out, would seem to be well beyond the scope of this methods paper and is, indeed, part of the ongoing work of improving and characterizing gel systems.

Moreover, bands in "typical" lanes 2, 3 and 7 are not found at the same height of those of lane 1 and not marked. At this stage, with a method proposed as a fast alternative to others, it is necessary to include a molecular marker in gels. 2D green/SDS PAGE should have been done to confirm attribution (although in line 356-7 it is written that these thylakoid samples are "dirty").

We have added lines to help indicate band identity. While band identification has been made based on 2D-SDS-PAGE, silver staining, Western Blotting, and MS, a presentation of such data is outside of the scope of this manuscript.

A comparative description of curves as in Fig. 2C is necessary to drive use of TCSPC: what types of decay are these? How can they be treated analytically? What values can one expected for a certain complex? How can they help decide if a band is a true supercomplex or a co-migration of disconnected complexes? How an TCSPC help resolve the central problem explained in lines 102-105?

We have attempted to make the answers to these questions more transparent in the text as they relate to the description of the method, although a thorough treatment of these questions would require a much more in depth discussion of the specific Biology involved, which again appears to be outside of the scope of the paper as desired by JoVE.

Comments about changes in shape of curves in Fig. 3 are not self-evident; the authors could add normalized versions of these graphs.

We have attempted to make these observations more specific.