**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 qI (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 qI. 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.