Seeing Beyond the Blot: A Critical Look at Assumptions and Raw Data Interpretation in Western Blotting

Maxwell DeNies[1], Allen Liu[2], and Santiago Schnell[1]

[1]University of Michigan Medical School
[2]University of Michigan College of Engineering

March 08, 2024

Abstract

Rapid advancements in technology refine our understanding of intricate biological processes, but a crucial emphasis remains on understanding the assumptions and sources of uncertainty underlying biological measurements. This is particularly critical in cell signaling research, where a quantitative understanding of the fundamental mechanisms governing these transient events is essential for drug development, given their importance in both homeostatic and pathogenic processes. Western blotting, a technique developed decades ago, remains an indispensable tool for investigating cell signaling, protein expression, and protein-protein interactions. While improvements in statistical analysis and methodology reporting have undoubtedly enhanced data quality, understanding the underlying assumptions and limitations of visual inspection in western blotting can provide valuable additional information for evaluating experimental conclusions. Using the example of agonist-induced receptor post-translational modification, we highlight the theoretical and experimental assumptions associated with western blotting and demonstrate how raw blot data can offer clues to experimental variability that may not be fully captured by statistical analyses and reported methodologies. This article is not intended as a comprehensive technical review of western blotting. Instead, we leverage an illustrative example to demonstrate how assumptions about experimental design and data normalization can be revealed within raw data and subsequently influence data interpretation.

Seeing Beyond the Blot: A Critical Look at Assumptions and Raw Data Interpretation in Western Blotting

Maxwell S. DeNies [a], Allen P. Liu [a,b], Santiago Schnell [c, d,*]

[a] Cellular and Molecular Biology Graduate Program, University of Michigan Medical School, Ann Arbor, Michigan, USA

[b] Department of Mechanical Engineering, University of Michigan, Ann Arbor, Michigan, USA

[c] Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana, USA

[e] Department of Applied & Computational Mathematics & Statistics, University of Notre Dame, Notre Dame, Indiana, USA

[*] Address correspondence to: Santiago Schnell (santiago.schnell@nd.edu)

Manuscript type: Review

Running head title: Seeing Beyond the Blot

Abbreviations used: PTMs, post-translational modifications

Keywords: rigor; reproducibility; undergraduate and graduate research education; experimental design; biometrology; quantitative biology; western blots; protein abundance; post-translational modification; cell signaling

DECLARATIONS

Funding: MSD thanks support from the National Science Foundation (Fellow ID: 2015196825).

Conflict of interest: The authors declare that they have no conflict of interest.

Data availability: The datasets generated/analyzed to illustrate the point of this essays are available from the Dr. Allen Lui (e-mail: allenliu@umich.edu) on reasonable request.

ABSTRACT

Rapid advancements in technology refine our understanding of intricate biological processes, but a crucial emphasis remains on understanding the assumptions and sources of uncertainty underlying biological measurements. This is particularly critical in cell signaling research, where a quantitative understanding of the fundamental mechanisms governing these transient events is essential for drug development, given their importance in both homeostatic and pathogenic processes. Western blotting, a technique developed decades ago, remains an indispensable tool for investigating cell signaling, protein expression, and protein-protein interactions. While improvements in statistical analysis and methodology reporting have undoubtedly enhanced data quality, understanding the underlying assumptions and limitations of visual inspection in western blotting can provide valuable additional information for evaluating experimental conclusions. Using the example of agonist-induced receptor post-translational modification, we highlight the theoretical and experimental assumptions associated with western blotting and demonstrate how raw blot data can offer clues to experimental variability that may not be fully captured by statistical analyses and reported methodologies. This article is not intended as a comprehensive technical review of western blotting. Instead, we leverage an illustrative example to demonstrate how assumptions about experimental design and data normalization can be revealed within raw data and subsequently influence data interpretation.

INTRODUCTION

The ability to accurately measure biological processes and quantify their inherent uncertainty is fundamental to scientific progress. Cell biology, in particular, presents unique challenges as measurements span multiple scales. For instance, intracellular protein concentrations of specific proteins can range from pM to nM, length of cellular structures range from nm to μm, forces generated by molecular motors in the pN range, and timescale of signaling transduction events from sub-seconds to hours. Biological systems also interact in complicated ways with their internal and external environment, and are dynamically changing making specific attributes difficult to measure. Consequently, biological measurements often yield a distribution of values. Despite technical advancements in -omics approaches as well technical and analytical improvements in microscopy, flow cytometry, and Western blotting, much of our understanding of molecular mechanisms remains primarily semi-quantitative.

A complete biological measurement requires both a value and an assessment of its associated uncertainty (1,2). While uncertainty is commonly defined as the experimental variability in a measurement between replicates, in theory it is additive of all uncertainties throughout the experimental process. The Guide to the Expression of Uncertainty in Measurement highlights the incomplete definition of the measurand (the quantity being measured) and the underlying assumptions as critical sources of uncertainty (3). Additionally, the possibility of non-representative or incomplete sampling of what was intended to be measured as well as an incomplete definition of the measurand are common sources of uncertainty defined within the Guide to the Expression of Uncertainty in Measurement. These sources are highly relevant in biological systems but can be harder to quantify. Understanding the inherent limitations within any biological measurement is crucial for both experimentalists and theorists when interpreting results.

Despite the emergence of alternative techniques to detect protein abundance, post-translational modifications (PTMs), and cell signaling – flow cytometry, FRET biosensors, microscopy, etc. (4–8) – Western blotting remains a widely used and accessible tool across biological disciplines (9). Quantitative biologists extract quantitative values from Western blots to parameterized models (see, (10), as an example). While improved statistical analyses and the inclusion of

individual data points offers transparency, carefully examining raw data, particularly for Western blots or immunofluorescence images, can provide valuable insights for interpretation.

In this perspective, we examine several factors that can influence Western blot analysis and interpretation. We illustrate these considerations using an example from our research (FIGURE 1), which investigated the impact of CXCR4 (C-X-C chemokine receptor 4) mutations (S338/39A and S324/25A) on CXCR4 expression and downstream signaling. In addition to probing for total protein and GAPDH (loading controls), we employed two antibodies to detect CXCR4: the commercial monoclonal antibody UMB2 and a polyclonal MYC antibody targeting an incorporated MYC epitope tag (FIGURE 1). It is important to note that UMB2, initially described as detecting total CXCR4, has subsequently been shown to exhibit sensitivity to CXCR4 PTMs (11–13). These experiments were conducted in RPE cells overexpressing individual CXCR4 constructs with a C-terminal MYC tag (WT), as wildtype RPE cells have negligible endogenous CXCR4 expression and are unresponsive to CXCL12 (a CXCR4 agonist) (7,12). In the following sections, we discuss how loading controls, experimental timescales, antibody stripping, and antibody banding patterns can shape Western blot interpretation.

This perspective does not aim to provide a comprehensive technical review of Western blotting; several excellent articles offer in-depth guidance on best practice (14–22). Instead, we focus on assumptions and aspects of Western blotting that should be considered by both theorists and experimentalists when evaluating data.

EXAMINING LOADING CONTROLS AND SIGNAL SATURATION IN WESTERN BLOTTING TO SEE IF EXPERIMENTAL CONDITIONS ARE COMPERABLE

Western blot analysis necessitates careful consideration of loading controls and signal linearity for accurate quantitative interpretation. While housekeeping proteins like GAPDH and actin remain widely used as loading controls, their expression levels can fluctuate across experimental conditions (23,24,15). In the case of FIGURE 1, while loading controls appear similar within each signaling time course, WT and mutant CXCR4 (S338/39A and S324/25A) samples are different. While not ideal, loading protein abundance variability is relatively common in experiments where multiple cell lines or +/- protein knockdown are compared due to unaccounted factors

such as differences in cell growth. This highlights the importance of confirming that the chosen loading control remains stable under your specific experimental treatments. Ideally, total protein staining should be used to normalize protein loading (25–27,15). This practice offers a more reliable representation of overall protein abundance.

To ensure equal loading, it is crucial to quantify protein concentrations prior to Western blotting. When comparing multiple cell lines or working with protein knockdowns, natural differences in cell growth or other unaccounted factors can lead to variations in protein abundance. Documenting protein concentrations alongside Western blots can help distinguish whether observed differences result from experimental treatments or variations in cell health/growth.

It is also important to ensure that antibody detection falls within the linear range of the chosen detection method. Oversaturated Western blot bands can lead to inaccurate quantification (15,17). Prior antibody titrations with various dilutions tested against a range of sample protein loads will establish optimal antibody concentrations for your experimental conditions. Image acquisition systems and freely available software like *ImageJ* can aid in the detection of signal saturation.

When working towards quantitative comparisons, the most robust approach is to analyze the slopes obtained from linear regression analysis of the integrated optical density values of immunoreactive bands across a range of increasing protein loads. This analysis should focus solely on values within the linear range. Slope ratio comparisons between samples offer greater reliability by correcting for inaccuracies in protein determination. This allows for more precise conclusions regarding increases or decreases in protein expression compared to qualitative band intensity assessments.

Key Takeaways

- Traditional housekeeping proteins (GAPDH, actin) can be unreliable.
- Opt for total protein staining whenever possible for more accurate normalization.
- Verify your chosen loading control remains stable under all experimental conditions.
- Quantify protein concentrations before Western blotting to ensure equal loading.

- Document protein concentrations alongside blots for informed interpretation.
- Perform antibody titrations to determine the linear range of detection.
- Use image analysis tools to check for saturation; oversaturated bands lead to inaccurate quantification.
- Analyze slopes from integrated optical density across protein loads for the most precise comparisons.

ADDRESSING POST-TRANSLATIONAL MODIFICATIONS AND ANTIBODY DETECTION IN TIME-SENSITIVE EXPERIMENTS

In time-sensitive experiments, such as those investigating signaling mechanisms, it is crucial to consider the potential impact of rapid protein modifications on antibody-based quantification. This section highlights the importance of understanding antibody recognition dynamics, especially within the context of PTMs.

The provided example using the UMB2 antibody to detect CXCR4 illustrates this point (FIGURE 1). While initially believed to detect total CXCR4, a significant decrease in detection following agonist stimulation suggests otherwise. This rapid change is unlikely due to protein degradation, nor is it explained by a shift in intracellular localization, as detection with an alternative MYC antibody remains consistent.

The most likely explanation lies in agonist-induced CXCR4 PTMs. These modifications, well-documented in the literature (25,28,29,12), can occur within the UMB2 antibody's epitope region and hinder its ability to bind CXCR4. While the UMB2 antibody remains a valuable tool (11–13), this example underscores the importance of understanding how PTMs or other alterations can influence antibody detection and, subsequently, data interpretation.

To illustrate the impact of mutations on antibody detection, let us examine the Western blot lanes (FIGURE 1; lanes 5-12). CXCR4 S338/39A and CXCR4 S324/25A are phospho-null mutant receptors of biologically relevant serine residues that regulate CXCR4 internalization and signaling (29). As described earlier, even minor mutations (in this case, just two residues) can greatly influence CXCR4 detection with the UMB2 antibody. This is evident in FIGURE 1. We

found that at the 0 min time point S338/39A mutant UMB2 detection was significantly higher than WT, while the S324/25A mutant UMB2 detection was negligible. Superficially, this could suggest that S338/39A increases CXCR4 expression while the S324/25A mutant is poorly expressed. However, investigation again with the MYC antibody clarifies that the S324/25A mutant is expressed (FIGURE 1; lanes 9-12).

Again, there are several hypotheses that may explain why this occurs. It is possible that S324/25A completely prevents CXCR4 UMB2 antibody detection due to a change in structure or leads to a different CXCR4 PTM state within the UMB2 epitope that decreases antibody affinity. In contrast, S338/39A detection with both the UMB2 and MYC antibody was elevated compared to WT CXCR4 suggesting that these mutations may have attenuated degradation and possibly PTM (FIGURE 1).

Further comparisons of how agonist-induced PTM is influenced by CXCR4 mutation further highlights the difficulty of solely relying on these data for interpretation as incomplete stripping differentially impacts the MYC detection of each of these receptors and decoupling of receptor mutation from CXCR4 detectability is not possible in this data alone (FIGURE 1). These are important questions that should be considered when evaluating western blots and can only be fully addressed through careful consideration of raw data and potentially additional experiments. In this case, the interpretation of the presented results is confounding and additional lines of evidence are necessary for a definitive conclusion.

Key takeaways

- Post-translational modifications (PTMs) can profoundly impact antibody detection dynamics.
- Rapid PTM changes can complicate quantification in time-sensitive experiments.
- Understanding how PTMs or other alterations might affect your antibody's target epitope is crucial for accurate data interpretation.
- Mutations can significantly influence PTM states and subsequent antibody recognition.
- Careful data analysis is required to distinguish between the effects of mutations on protein expression versus detectability.

- Incomplete antibody stripping can further complicate interpretation; raw data inspection is necessary to identify potential issues.

CONSIDER HOW WESTERN BLOT STRIPPING CAN INFLUENCE DATA QUANTIFICATION

Incomplete antibody stripping is a common issue in Western blotting, particularly when evaluating signaling cascades using phospho-specific antibodies. To accurately quantify both phosphorylated and total protein levels on the same blot, it is necessary to effectively remove the phospho-specific antibody prior to reprobing. Failure to do so can introduce residual signal, leading to inaccurate quantification. FIGURE 2 demonstrates this with ERK1/2 phosphorylation: even after stripping, residual phospho-ERK1/2 signal persists. In this experiment, we monitored CXCL12-induced ERK1/2 phosphorylation in RPE cells overexpressing WT or mutant CXCR4. The western blot was imaged both pre- and post-antibody stripping. As expected prior to antibody stripping, CXCL12 robustly induces ERK1/2 phosphorylation (Figure 2). However, while less pronounced, phospho-ERK1/2 staining persists after antibody stripping. Subsequent staining with total ERK1/2 antibody of the same species or a similarly sized protein could lead to inaccurate quantification.

One solution is to use total protein antibodies raised in a different species, eliminating the need for stripping. However, limited resources and antibody availability can make this challenging. Even when using primary antibodies raised in different hosts, stripping is necessary when working with Horseradish peroxidase (HRP)-conjugated secondaries. This is essential to prevent cross-reactivity between the HRP-conjugated secondary antibodies. While effective stripping is a standard technique for laboratories routinely using Western blotting, various homemade buffers exist alongside commercial options. The choice depends on your specific antibodies and experimental needs. While imperfect, stripping and reprobing is often necessary and may not change overall data interpretation.

One example of this is shown in FIGURE 1 lines 1-4, which illustrates how residual primary antibody can impact quantification. UMB2 detection is negatively correlated with CXCR4 PTM.

Therefore, in this scenario the MYC antibody is the total signaling protein antibody and UMB2 is PTM sensitive. The MYC antibody (total protein) shows a visible decrease in detection at later time points compared to UMB2 (PTM-sensitive). This is not biologically relevant as we have previously confirmed that CXCR4 is not degraded or differentially extracted at the 20 min stimulus time point (12). Since CXCR4 is stable at these time points, this decrease is likely due to residual UMB2 antibody signal detected during MYC quantification. This highlights the potential for underestimating PTMs in such cases.

To assess the extent of incomplete stripping, reprobe the freshly stripped blot with secondary antibody alone. Residual primary antibodies will produce a similar banding pattern to the pre-stripped blot and can influence quantification, particularly when comparing WT and mutant proteins or knockdowns.

Key Takeaways

- Incomplete antibody stripping can impact Western blot quantification, particularly in phospho-signaling studies and comparisons across experimental conditions.
- Using primary antibodies of different species can mitigate stripping issues, though resource constraints may limit this approach.
- Reprobing a stripped blot with secondary antibody alone can help assess stripping efficacy.
- Careful inspection of raw Western blot data is crucial for understanding potential limitations and accurate data interpretation.

EVALUATE ANTIBODY BANDING PATTERNS TO IDENTIFY POTENTIAL QUANTIFICATION LIMITATIONS

Careful analysis of antibody banding patterns in Western blots can reveal valuable information about potential limitations in quantification. An important principle is that phospho-specific antibodies should detect a subset of bands detected by the corresponding total protein antibody. This reflects the fact that only a portion of the total protein is usually phosphorylated at any

given time. For instance, phospho-ERK1/2 antibodies should detect a subset of bands visualized by the total ERK1/2 antibody.

Deviation from this pattern can signal issues with antibody specificity or experimental conditions that could affect quantification. In FIGURE 1, the UMB2 antibody (PTM-sensitive) detects bands within a subset of bands recognized by the MYC antibody (total protein), demonstrating consistency with this principle. While visual inspection of bands offers preliminary insights, it is essential to remember that apparent differences in band intensity may not directly translate to actual changes in protein abundance. Densitometric analysis of Western blot images is crucial for reliable quantification.

To minimize these timing discrepancies, particularly when comparing multiple samples, steps like media removal and cell lysis should be performed on ice wherever possible. Precision technologies like microfluidics offer another avenue for more accurate control over the timing of stimulus and lysis.

The caveats described in this section, while focusing on CXCR4 PTM, illustrate principles applicable to other Western blotting experiments and even related techniques like immunofluorescence microscopy.

Key Takeaways

- Consider both antibody banding patterns and imaging sensitivity when interpreting Western blot results.
- Visual band intensity differences may not always reflect true biological changes.
- Densitometric analysis provides a more reliable quantitative assessment of Western blot data, especially when comparing samples with variations in signal strength.

HOW DO WESTERN BLOTTING ASSUMPTIONS INFLUENCE QUANTIFICATION?

The assumptions we rely on when interpreting Western blots can significantly impact quantification and data interpretation. Here, we illustrate how these assumptions, particularly

those around data normalization, can influence interpretation. Reliable quantification depends on working within the linear range of antibody detection, ensuring accurate comparisons between samples.

The first step in quantification is data normalization, essential to account for technical and experimental variability. Let us consider a hypothetical experiment (FIGURE 3A), comparing control and treatment groups (e.g., inhibitor, knockdown, mutant protein) at 0- and 5-minutes post-stimulus. Normalization strategies can have a significant impact in quantification:

- **Normalizing to the initial pre-treatment sample (FIGURE 3B (I)):** Pre-treatment signals often have lower signal-to-noise ratios. Minor variations, when used as the basis of normalization, can significantly amplify uncertainty in normalized values, especially if signals fall outside the linear detection range.
- **Normalizing to the maximum value for each group (FIGURE 3B (II)):** This mitigates uncertainty amplification but prevents direct comparison of maximum responses between different experimental groups.
- **Normalizing to the control's maximum value (FIGURE 3B (III)):** This reduces inter-blot variability and generally offers better signal-to-noise ratios. It also allows for comparisons between treatments' maximum responses. However, this approach assumes that the treatment does not significantly impact overall protein expression or other signaling components.

The above examples highlight the importance of signals falling within the linear detection range for reliable quantification. There are two preferred methods for accurate quantitation. First, assessing linearity by determining the linear range for each antibody through antibody titrations across a range of protein loads. Subsequent analyses should use values exclusively within this range. Second, slope analysis by comparing slopes obtained by linear regression analysis of integrated optical density for a range of protein loads provides the most robust quantitative approach.

Key Takeaways

- The choice of normalization strategy can influence results and interpretation.
- Working within the linear range of your detection method is essential for accurate quantification.
- Transparency in reporting normalization methods, along with explicit descriptions in figure legends and methodology sections, is crucial.

KEY CONSIDERATIONS FOR INTERPRETING CELL SIGNALING EXPERIMENTS IN THE CONTEXT OF WESTERN BLOTTING

The complexity of cellular signaling pathways underscores the need for careful interpretation of Western blot data, particularly when interventions like knockdowns or mutations are involved. Here are some key considerations:

- **Compensatory Mechanisms:** Cells often have multiple pathways regulating receptor signaling and endocytosis. Knockdowns or mutations can trigger compensatory mechanisms, obscuring the direct effects of your intervention. Whenever possible, design experiments to decouple primary effects from potential compensatory changes.
- **Protein Multifunctionality:** Many proteins involved in endocytic trafficking have multiple roles and localizations within the cell (30,31). Changes in protein levels or mutations can have broader consequences beyond their intended target. Consider potential secondary effects when interpreting results.
- **Cellular and Experimental Timescales:** When interpreting signaling data with short timescales, it is essential to consider the practical limitations of experimental procedures. Assume that it takes approximately 30 seconds to take cells from the incubator, remove media, and prep samples for protein extraction. While a Western blot might indicate a "1-minute stimulus", in reality is closer to 1.5 minutes – a 50% increase in stimulus time. This variability might be less significant at later time points but can be a concern for early time points in signaling cascades.
- **Receptor Regulation:** Signal transduction pathways are tightly regulated. Changes in receptor localization, expression levels, or PTMs due to experimental manipulations can influence downstream signaling in ways that might complicate quantification and interpretation (32,33).

- **Spare Receptor Hypothesis:** Often, only a fraction of receptors need to be activated for maximal signaling responses (34). This is important to consider when changes in receptor expression or trafficking are part of the experimental design.
- **Methodological Rigor:** Whenever possible, use multiple, complementary approaches to confirm your conclusions from Western blots analysis (35,36). This could involve microscopy-based techniques to assess localization or alternative signaling readouts.

CONCLUSION

Scientific innovation thrives on creativity, the advancement of technologies, and the continuous refinement of research methodologies. However, within this dynamic environment, there's a heightened potential for honest yet irreplicable results. This lack of replicate stems from a multitude of factors, including insufficient standardization in reporting experimental protocols, flawed experimental design, statistical challenges, or biases in hypothesis testing. In this work, we highlight another crucial factor influencing replicate: the fundamental assumptions underlying the biological measurements themselves. We believe that incorporating principles of metrology, the science of measurement, within receptor signaling could significantly reduce the issues of replicability, and importantly, guide discoveries toward reaching scientific results with rigor.

Throughout this article, we have revisited the core assumptions of Western blotting, emphasizing important considerations that contribute to data uncertainty. As biology transitions toward a more quantitative field, achieving replicability between different research groups becomes the cornerstone of scientific rigor. This demands greater responsibility across the scientific community to uphold gold standards for reporting measurement protocols and associated assumptions. While these standards will not impede novel or significant findings, they will ensure that research results align with the presented conclusions. Additionally, we must develop standardized methods and protocols for sharing measurements. While our focus was on Western blotting, many of these core concepts extend to other methodologies commonly used in cell signaling research and cell biology in general.

ACKNOWLEDGEMENTS

We would like to thank Dr. Justin Eilertsen (University of Michigan), Dr. Wylie Stroberg (University of Alberta) and Hesso Farhan (Medical University of Innsbruck) for constructive discussions.

REFERENCES

1. Plant AL, Locascio LE, May WE, Gallagher PD. Improved reproducibility by assuring confidence in measurements in biomedical research. Nat Methods. 2014 Sep;11(9):895–8.

2. Plant AL, Becker CA, Hanisch RJ, Boisvert RF, Possolo AM, Elliott JT. How measurement science can improve confidence in research results. PLoS Biol. 2018 Apr;16(4):e2004299.

3. BIPM. BIPM - Guide to the Expression of Uncertainty in Measurement (GUM) [Internet]. 2008 [cited 2018 Oct 12]. Available from: https://www.bipm.org/en/publications/guides/gum.html

4. Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, Davis RJ, et al. A genetically encoded fluorescent sensor of ERK activity. Proc Natl Acad Sci. 2008 Dec 9;105(49):19264–9.

5. Malik R, Soh UJK, Trejo J, Marchese A. Novel Roles for the E3 Ubiquitin Ligase Atrophin-interacting Protein 4 and Signal Transduction Adaptor Molecule 1 in G Protein-coupled Receptor Signaling. J Biol Chem. 2012 Mar 16;287(12):9013–27.

6. Miura H, Matsuda M, Aoki K. Development of a FRET biosensor with high specificity for Akt. Cell Struct Funct. 2014;39(1):9–20.

7. Davies R, Vogelsang P, Jonsson R, Appel S. An optimized multiplex flow cytometry protocol for the analysis of intracellular signaling in peripheral blood mononuclear cells. J Immunol Methods. 2016 Sep 1;436:58–63.

8. Eichel K, Zastrow M von. Subcellular Organization of GPCR Signaling. Trends Pharmacol Sci. 2018 Feb 1;39(2):200–8.

9. Burnette WN. Western blotting : remembrance of past things. Methods Mol Biol Clifton NJ. 2009;536:5–8.

10. Wynn ML, Egbert M, Consul N, Chang J, Wu ZF, Meravjer SD, et al. Inferring Intracellular Signal Transduction Circuitry from Molecular Perturbation Experiments. Bull Math Biol. 2018 May;80(5):1310–44.

11. Mimura-Yamamoto Y, Shinohara H, Kashiwagi T, Sato T, Shioda S, Seki T. Dynamics and function of CXCR4 in formation of the granule cell layer during hippocampal development. Sci Rep. 2017 Jul 17;7(1):5647.

12. DeNies MS, Rosselli-Murai LK, Schnell S, Liu AP. Clathrin Heavy Chain Knockdown Impacts CXCR4 Signaling and Post-translational Modification. Front Cell Dev Biol. 2019;7:77.

13. DeNies MS, Smrcka AV, Schnell S, Liu AP. β-arrestin mediates communication between plasma membrane and intracellular GPCRs to regulate signaling. Commun Biol. 2020 Dec 18;3(1):1–12.

14. Taylor SC, Berkelman T, Yadav G, Hammond M. A Defined Methodology for Reliable Quantification of Western Blot Data. Mol Biotechnol. 2013 Nov;55(3):217–26.

15. Eaton SL, Hurtado ML, Oldknow KJ, Graham LC, Marchant TW, Gillingwater TH, et al. A Guide to Modern Quantitative Fluorescent Western Blotting with Troubleshooting Strategies. J Vis Exp JoVE [Internet]. 2014 Nov 20;(93). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4354265/

16. Ghosh R, Gilda JE, Gomes AV. The necessity of and strategies for improving confidence in the accuracy of western blots. Expert Rev Proteomics. 2014 Oct;11(5):549–60.

17. Janes KA. An analysis of critical factors for quantitative immunoblotting. Sci Signal. 2015 Apr 7;8(371):rs2–rs2.

18. Bell G. Quantifying western blots: none more black. BMC Biol. 2016 Dec;14(1):116.

19. Bass JJ, Wilkinson DJ, Rankin D, Phillips BE, Szewczyk NJ, Smith K, et al. An overview of technical considerations for Western blotting applications to physiological research. Scand J Med Sci Sports. 2017 Jan;27(1):4–25.

20. Kirshner ZZ, Gibbs RB. Use of the REVERT® total protein stain as a loading control demonstrates significant benefits over the use of housekeeping proteins when analyzing brain homogenates by Western blot: An analysis of samples representing different gonadal hormone states. Mol Cell Endocrinol. 2018 Sep;473:156–65.

21. Butler TAJ, Paul JW, Chan EC, Smith R, Tolosa JM. Misleading Westerns: Common Quantification Mistakes in Western Blot Densitometry and Proposed Corrective Measures. BioMed Res Int. 2019 Jan 21;2019:1–15.

22. Pillai-Kastoori L, Schutz-Geschwender AR, Harford JA. A systematic approach to quantitative Western blot analysis. Anal Biochem. 2020 Mar;593:113608.

23. Ferguson RE, Carroll HP, Harris A, Maher ER, Selby PJ, Banks RE. Housekeeping proteins: A preliminary study illustrating some limitations as useful references in protein expression studies. PROTEOMICS. 2005 Feb;5(2):566–71.

24. Li R, Shen Y. An old method facing a new challenge: Re-visiting housekeeping proteins as internal reference control for neuroscience research. Life Sci. 2013 Apr;92(13):747–51.

25. Dittmer A, Dittmer J. β-Actin is not a reliable loading control in Western blot analysis. ELECTROPHORESIS. 2006 Jul 1;27(14):2844–5.

26. Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ. The use of total protein stains as loading controls: An alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. J Neurosci Methods. 2008 Jul 30;172(2):250–4.

27. Gilda JE, Gomes AV. Stain-Free total protein staining is a superior loading control to β-actin for Western blots. Anal Biochem. 2013 Sep 15;440(2):186–8.

462 28. Busillo JM, Armando S, Sengupta R, Meucci O, Bouvier M, Benovic JL. Site-specific
463 Phosphorylation of CXCR4 Is Dynamically Regulated by Multiple Kinases and Results in
464 Differential Modulation of CXCR4 Signaling. J Biol Chem. 2010 Mar 5;285(10):7805–17.

465 29. Luo J, Busillo JM, Stumm R, Benovic JL. G Protein-Coupled Receptor Kinase 3 and Protein
466 Kinase C Phosphorylate the Distal C-Terminal Tail of the Chemokine Receptor CXCR4 and
467 Mediate Recruitment of β-Arrestin. Mol Pharmacol. 2017;91(6):554–66.

468 30. Liberali P, Pelkmans L. Nature Cell Biology. 2012 [cited 2018 Oct 8]. Towards quantitative
469 cell biology. Available from: https://www.nature.com/articles/ncb2648

470 31. Thul PJ, Åkesson L, Wiking M, Mahdessian D, Geladaki A, Ait Blal H, et al. A subcellular
471 map of the human proteome. Science. 2017 26;356(6340).

472 32. Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, et al. Clathrin-
473 independent endocytosis of ubiquitinated cargos. Proc Natl Acad Sci U S A. 2005 Feb
474 22;102(8):2760–5.

475 33. Sigismund S, Algisi V, Nappo G, Conte A, Pascolutti R, Cuomo A, et al. Threshold-
476 controlled ubiquitination of the EGFR directs receptor fate. EMBO J. 2013 Jul
477 31;32(15):2140–57.

478 34. Brown L, Deighton NM, Bals S, Söhlmann W, Zerkowski HR, Michel MC, et al. Spare
479 receptors for beta-adrenoceptor-mediated positive inotropic effects of catecholamines in the
480 human heart. J Cardiovasc Pharmacol. 1992 Feb;19(2):222–32.

481 35. Kaelin Jr WG. Publish houses of brick, not mansions of straw. Nat News. 2017 May
482 25;545(7655):387.

483 36. Munafò MR, Smith GD. Robust research needs many lines of evidence. Nature. 2018
484 Jan;553(7689):399.

485

Figures

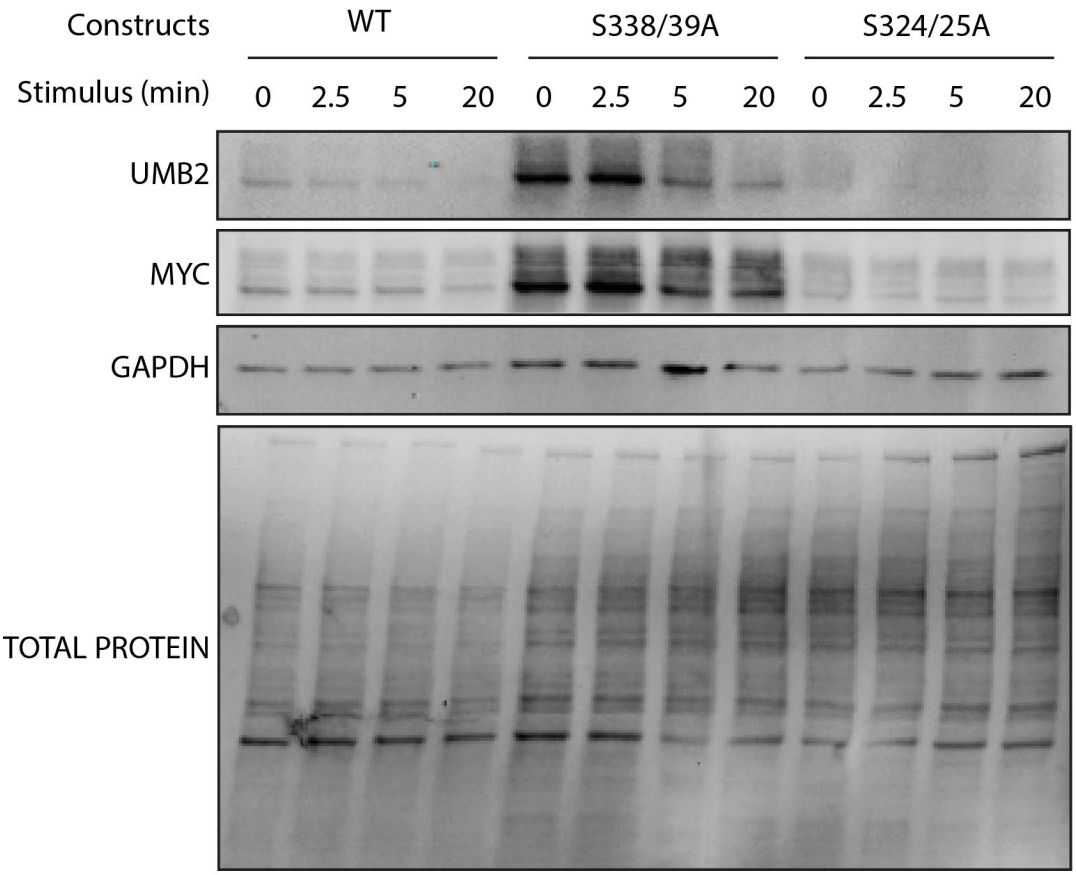

Figure 1: Western blotting assumptions are not always true. Representative western blot of non-post-translationally modified (UMB2) and total CXCR4 (MYC) detection for WT RPE cells transferred with CXCR4 (WT) and serine mutant receptors (S338/39A and S324/25A). Total Protein and GAPDH staining illustrate protein loading for each replicate. **Experimental methods:** RPE cells overexpressing CXCR4 were grown to 75% confluence a treated with fresh FBS (10%) supplemented media (Gibco PN: 11330-032) 24 hours before the experiment. Cells were serum starved for 4 hours and treated with 12.5 nM CXCL12 (R&D Systems PN: 350-NS-050) for the described time course. Lysates were extracted using RIPA buffer (Pierce PN: 89900) supplemented with protease and phosphatase inhibitors (Thermo Scientific PN: and respectively) and incubated ice for 20 minutes and centrifuged at 16,100g for 45 minutes at 4°C. Loading buffer supplemented with beta mercaptoethanol was added to denature lysates and samples were loaded on a 4-20% BioRad gel and transferred onto PVDF membranes using the iBlot system

mixed range transfer (Thermo Scientific). Total protein was quantified using the REVERT Total Protein Stain (LiCor PN: 926-11016). Afterwards, blots were blocked with 5% BSA (Thermo Scientific PN: 37520) in TBST for 1 hour and incubated with primary antibodies ms-GAPDH (1:1000), rb-UMB2 (1:2000) overnight at 4°C. Blots were incubated with secondary antibodies (Gt anti ms-700, gt anti rb-800) for 1 hour in 5% BSA in TBST and imaged using the LiCor Odyssey SA Imaging System. Afterwards, blots were stripped using NewBlot stripping buffer (LiCor PN:928-40032) per manufacture instructions and reprobed with rb-MYC antibody (1:5000) as described above. UMB2 antibody was purchased from ABCAM (PN: Ab124824), MYC from Bethyl (PN: A190-105A) and GAPDH from Santa Cruz Biotechnology (PN: sc-47724). Secondary antibodies were purchased from Invitrogen (PN: SA535571 and 35518).

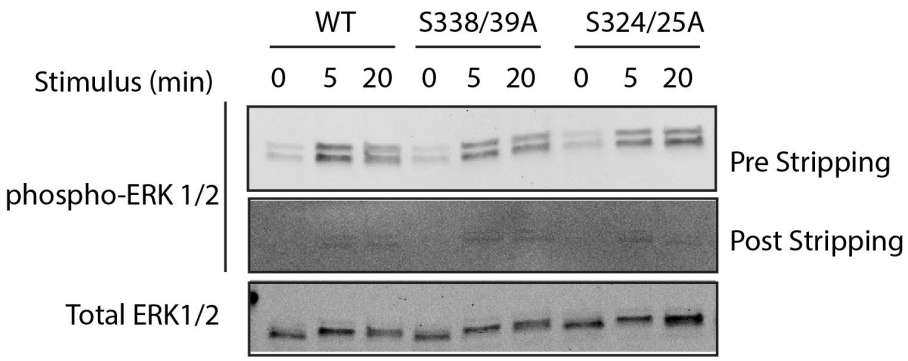

Figure 2: Example of incomplete western blot stripping. Phospho-ERK1/2 detection pre and post-antibody stripping. Total ERK1/2 staining is shown as a loading control. **Experimental methods:** RPE cells overexpressing WT or mutant CXCR4 were grown to 75% confluence a treated with fresh FBS (10%) supplemented media (Gibco PN: 11330-032) 24 hours before the experiment. Cells were serum starved for 4 hours and treated with 12.5 nM CXCL12 (R&D Systems PN: 350-NS-050) for the described time course. Lysates were extracted using RIPA buffer (Pierce PN: 89900) supplemented with protease and phosphatase inhibitors (Thermo Scientific PN: and respectively) and incubated ice for 20 minutes and centrifuged at 16,100g for 45 minutes at 4°C. Loading buffer supplemented with beta mercaptoethanol was added to denature lysates and samples were loaded on a 4-20% BioRad gel and transferred onto PVDF membranes using the iBlot system mixed range transfer (Thermo Scientific). Blots were blocked with 5% BSA (Thermo Scientific PN: 37520) in TBST for 1 hour and incubated with primary antibodies ms-Total-ERK1/2 (1:1000), rb-phospho-ERK1/2 (1:2000) in 5% BSA in TBST overnight at 4°C. Blots were incubated with secondary antibodies (Gt anti ms-700, gt anti rb-800) for 1 hour in 5% BSA in TBST and imaged using the LiCor Odyssey SA Imaging System. Afterwards, blots were stripped using NewBlot stripping buffer (LiCor PN:928-40032) and imaged again to determine antibody stripping efficacy. Total and phospho-ERK1/2 antibodies were purchased from Cell Signaling Technologies (PN: 9107S and 4370S respectively). Secondary antibodies were purchased from Invitrogen (PN: SA535571 and 35518).

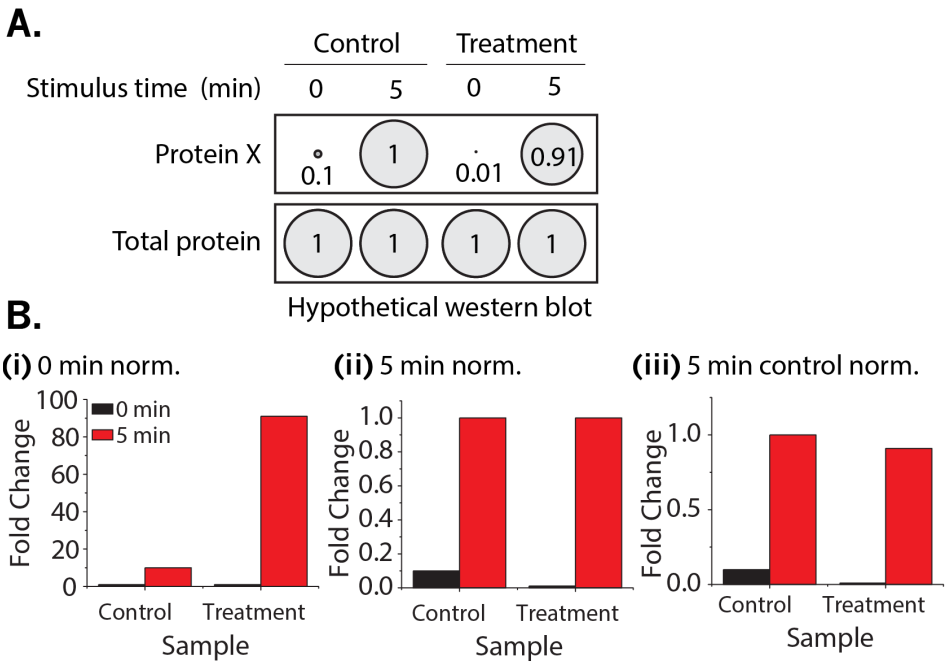

Figure 3: Normalization methodology can influence result interpretation. (**A**) Hypothetical western blot results for a control and treatment experiment. Circles diameters are representative of western blot band intensities and are listed. (**B**) Quantification of hypothetical western blot results illustrating that normalizing to samples with low signal to noise ratio can propagate error throughout normalization and influence result interpretation. For this representation, noise was assumed to be constant for each sample. (i) Hypothetical quantification of data when normalized to the 0 min time point of each treatment (i.e. normalizing value: 0.1 and 0.01 for the control and treatment respectively). (ii) Hypothetical quantification of data when normalized to 5-minute time point of each condition (i.e. normalizing value: 1 and 0.91 for the control and treatment respectively). (iii) Hypothetical quantification of data when normalized to the 5-minute time point of the control condition (i.e. normalizing value: one for all samples).