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Review

An overview of technical considerations for Western blotting applications to physiological research

J. J. Bass, D. J. Wilkinson, D. Rankin, B. E. Phillips, N. J. Szewczyk, K. Smith, P. J. Atherton

MRC/ARUK Centre of Excellence for Musculoskeletal Ageing Research, School of Medicine, University of Nottingham, Derby, UK
Corresponding author: Dr. Philip J. Atherton, MRC-ARUK Centre of Excellence for Musculoskeletal Ageing Research, School of Medicine, Derby DE22 3DT, UK. Tel: 01332 724725, Fax: 01332 724727, E-mail: philip.atherton@nottingham.ac.uk

The applications of Western/immunoblotting (WB) techniques have reached multiple layers of the scientific community and are now considered routine procedures in the field of physiology. This is none more so than in relation to skeletal muscle physiology (i.e., resolving the mechanisms underpinning adaptations to exercise). Indeed, the inclusion of WB data is now considered an essential aspect of many such physiological publications to provide mechanistic insight into regulatory processes. Despite this popularity, and due to the ubiquitous and relatively inexpensive availability of WB equipment, the quality of WB in publications and subsequent analysis and interpretation of the data can be variable, perhaps resulting in spurious conclusions. This may be due to poor laboratory technique and/or lack of comprehension of the critical steps involved in WB and what quality control procedures should be in place to ensure robust data generation. The present review aims to provide a detailed description and critique of WB procedures and technicalities, from sample collection through preparation, blotting and detection, to analysis of the data collected. We aim to provide the reader with improved expertise to critically conduct, evaluate, and troubleshoot the WB process, to produce reproducible and reliable blots.

The Western blot (WB) has diverse applications for investigating regulatory molecular events underpinning energy metabolism, protein turnover and chronic physiological adaptations. For example, the WB can be used to investigate protein abundance, kinase activity, cellular localization, protein–protein interactions, or monitoring of post-translational modifications [i.e., events of cleavage, phosphorylation (Nairn et al., 1982), ubiquitinylation (Paul et al., 2012), glycosylation (Péré-Brissaud et al., 2015), methylation (Voelkel et al., 2013), and SUMOylation (Park-Sarge & Sarge, 2010); to name the main applications]. While such WB approaches are routinely used in many fields of biochemical research, the application of the WB to skeletal muscle and exercise physiology is increasing. This is for reasons relating to the pursuit of an improved understanding of molecular pathways involved in the regulation of transcription and translation by exercise and nutrition in health, aging, and disease. This expansion in WB applications has led to an increased number of users lacking analytical biochemistry backgrounds to appreciate important caveats.

Crucial quality control elements of a WB may be overlooked, leading to poor quality blots, and the potential for unintentionally misleading data production and interpretation.

Outwardly, the principle of the WB is based around a few broad steps: (a) the extraction of cellular proteins from a complex mixture of intracellular and extracellular proteins (from tissue, cells, etc.); (b) quantification of protein concentration and electrophoretic separation of proteins within a gel matrix; (c) transfer to a membrane with a high affinity for proteins; (d) "blocking" the membrane to reduce non-specific binding; (e) antigen detection by antibodies specific for the protein(s) of interest; (f) incubation with a secondary antibody linked to a label (e.g., chemiluminescent or fluorescent); (g) development and detection of the signal, which is theoretically proportional to the degree of antigen/antibody binding; and (h) quantification of the resulting bands using densitometry software (Fig. 1). Originally, the process of "Western blotting" was the aspect of transferring proteins from a gel to a more stable membrane, although it commonly now refers to the whole process. To allow for the greatest accuracy and interpretation of data, each aspect of the WB process must be understood and carefully considered. In this review, we will describe the stages of the WB, focusing on the more routine WB gel
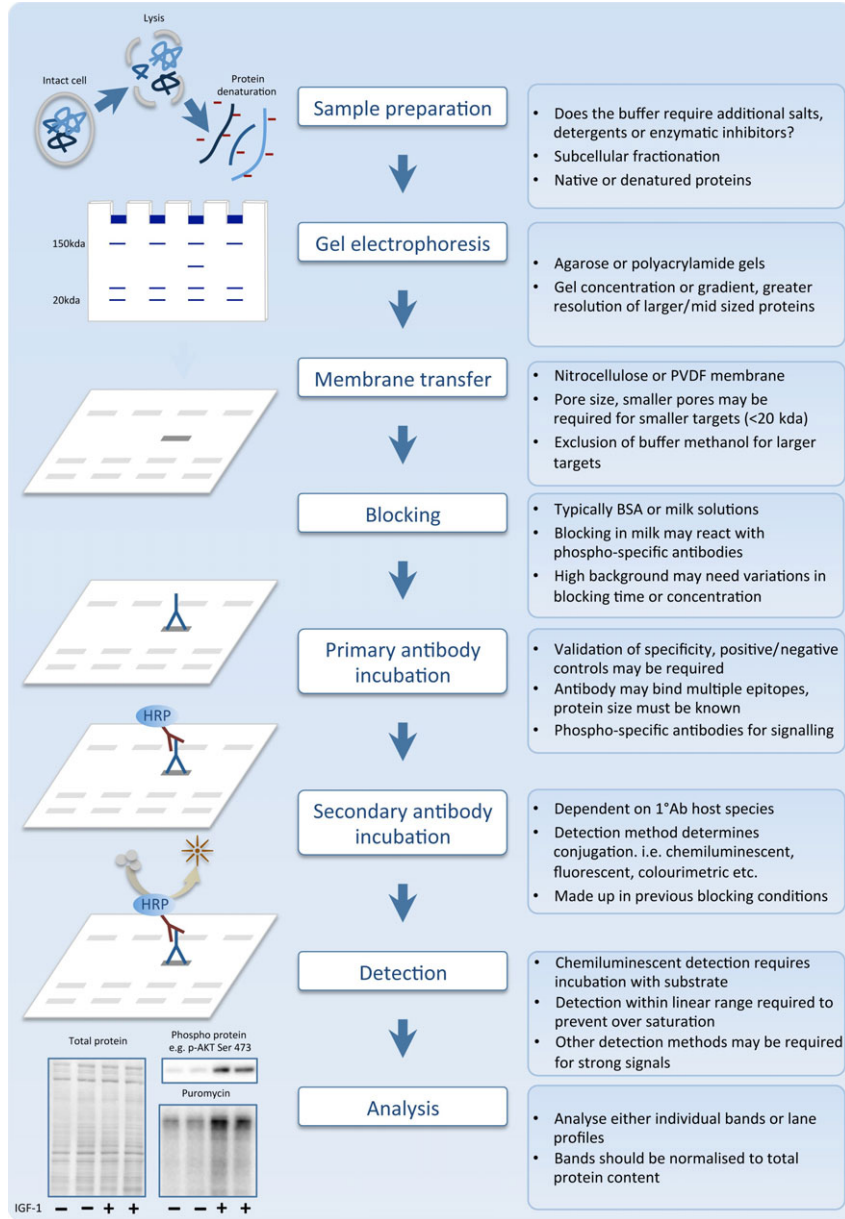
Fig. 1. The sequential stages of the Western blot process.

electrophoresis methodologies using standard SDS-PAGE, wet transfers, and chemiluminescence, critiquing and highlighting important points to consider throughout. In the main, we will concentrate on analysis of skeletal muscle tissues derived from skeletal muscle biopsies; nonetheless, the details described in each element are inherently applicable to other tissues or sample types. While performing a WB, there are multiple key aspects to each step:

Sample preparation:

- Is the target protein soluble/cytoplasmic, insoluble or membrane bound?

- Are additional buffer components (e.g., detergents, enzymatic inhibitors) required for solubilization, fractionation, or maintenance of post-translational modifications?
- What is the method of protein quantification, will buffer components interfere?

Polyacrylamide gel electrophoresis:

- What concentration of gel is most appropriate (e.g., 20% for proteins < 20 kDa, 7.5% for proteins > 200 kDa)?
- What running buffer is most suitable (e.g., MOPS for proteins ˜75 kDa, MES for proteins < 36 kDa)?

- How long and what voltage to run the tank (typically 60 min at 200 V)?

  Electro-transfer:

- What is the most suitable membrane material (e.g., PVDF or nitrocellulose) and pore size (e.g., 0.45 μm)?
- Is methanol excluded in the buffer (i.e., for transfer of larger proteins)?
- Should membrane staining occur to assess transfer efficiency (e.g., Ponceau)?

  Blocking:

- Which blocking reagent is most suitable (e.g., BSA or milk)?
- What concentration (e.g., 2.5%) and what buffer (e.g., TBST) should be used?

  Primary antibody:

- What are the general characteristics of the primary antibody (e.g., monoclonal Rabbit IgG)?
- Is it specific toward native or denatured proteins and is the epitope sequence/region know?
- Are additional bands known/present (i.e., degradation products or protein isoforms)?
- Have appropriate controls been run to determine specificity?
- Is the antibody specific to a post-translational modification (e.g., phosphorylation)?

  Secondary antibody:

- What is the label conjugation (e.g., HRP or fluorescent)?
- Is the secondary antibody specific toward the primary isotype?
- Is there detectable/overexposed signal, if so is an antibody dilution curve required?

  Detection:

- What is the detection method (e.g., chemiluminescent or fluorescent)?
- Is fluorescent multiplexing suitable?
- Have the antibodies been stored correctly?
- Can the membrane be successfully stripped and reprobed?

  Analysis and normalization:

- Is the band of interest within the linear range of the detection system?
- What method of quantification is most suitable (i.e., whole lane or boxed analysis)?
- What is the method of background detection (e.g., rolling ball algorithm)?
- What is the method of normalization (e.g., Coomassie stain)?
- Is it more suitable to measure the total vs phosphorylation expression of a protein?

Sample handling

The method of sample collection depends on the sample type: skeletal muscle tissue by biopsy [i.e., for human tissue typically by conchotome or Bergström needle (Dietrichson et al., 1987)], samples dissected post-mortem (i.e., after the terminal procedure of an *in vivo* experiment), and cell culture using scrapers (Quach et al., 2009). Once tissue samples are harvested, they should be immediately washed in an ice-cold neutral pH buffer, before removal of visible fat, snap frozen in liquid N_2, and stored at -80 °C. These steps are designed to limit protein degradation and preserve post-translational modifications (PTMs), while concurrently reducing non-skeletal muscle cell contaminants and blood, all of which could interfere with downstream processes (e.g., hemoglobin may interfere with colorimetric protein assays (Doumas et al., 1981) or contaminate muscle cell-specific analysis). In order to obtain robust data, samples should remain frozen until use and undergo as little manipulation as possible (e.g., periods at higher temperatures and multiple freeze thaws, so to minimize degradation as indicated by gel streaks) (Mahmood & Yang, 2012). Sometimes, it may be viable for fresh tissue to be utilized for WB sample preparation; however, it is more common and in our experience preferable to snap freeze tissues. This allows multiple analyses to be performed on a single sample (e.g., mass spectrometry, quantitative real-time PCR) while controlling the amount of tissue utilized. Although typically whole skeletal muscle tissue is used, it is possible to isolate and group fibers [i.e., based on fiber type (Jensen & Richter, 2011)] or use individual fibers for WB analysis (Murphy & Lamb, 2013). These techniques, however, are time consuming and may reduce the protein yield, but may be more informative depending on the experimental design (i.e., influence of fiber type on an intervention).

The extraction of proteins of interest from tissues requires the lysis and disruption of cell membranes using homogenization techniques, typically in the form of mechanical, sonication, and/or chemical approaches. For muscle biopsies or other solid tissues, mechanical homogenization is required to mince large sections of tissue and disrupt membranes (in order to liberate intracellular proteins). High-powered bench-top "polytron" homogenizers, commercial "bead-beaters", or simple scissor snipping may be used to effectively release intracellular proteins into solution (Goldberg, 2008). It is noteworthy that certain mechanical methods (e.g., polytron approaches) may retain remnants of tissues if not thoroughly cleaned, leading to cross-sample contamination. If protein yields are lower than expected after quantification (e.g., in our experience yields for

Table 1. Common buffer components for use in tissue homogenization

Chemical	Purpose	Typical concentration
Triton x-100	Increase solubility of non-polar proteins	0.1–1%
Urea	Disruption of protein hydrogen bonds increasing solubility	6–8 M
NaCl	Membrane disruption and protein solubility	1–100 mM
SDS	Membrane disruption	0.1–1%
Tris-HCl	Solution buffering	50 mM
MOPS/MES	Solution buffering	1–5 mM
HEPES	Solution buffering	50 mM
Glycerol	Solution stabilization	5–10%
EGTA/EDTA	Inhibition of metalloproteases and prevention of changes in protein phosphorylation	1 mM
Na_3VO_4	Tyrosine and alkaline phosphatase inhibition	0.5 mM
NaF	Ser/Thr phosphatase inhibition	50 mM
β-glycerophosphate	Ser/Thr phosphatase inhibition	10 mM
β-mercaptoethanol	Cleavage of disulfide bonds and protein denaturation	1–10 mM
DTT	Cleavage of disulfide bonds and protein denaturation	1–10 mM

cultured cells are typically 0.5–1.5 µg/µl, human skeletal muscle biopsies 3–6 µg/µl and excised rat kidney 12–15 µg/µl) (discussed below), sonication of samples might be required to further lyse cellular membranes. Sonication utilizes high frequency sound waves to disrupt cellular membranes, nonetheless, this should follow mechanical homogenization to remove large pieces of tissue that would not be disrupted by sonication alone (Autuori et al., 1982). Notably, homogenization of muscle cell cultures does not require the same degree of mechanical lysing; instead vigorously pipetting the collected cells through a small gauge syringe or fine tip gel-loading pipette is typically sufficient (Crossland et al., 2013).

Tissues should be homogenized in a buffer designed to solubilize and optimize preservation of the target proteins. Accurately buffering a homogenization solution proximate to the isoelectric point of proteins (the pH at which they have neutral charge), is necessary (pH 7–9) to ensure solubility, and prevention of protein precipitation, through maintenance of positive or negatively charged amino acid functional (R) groups (Grabski, 2009). Addition of non-ionic detergents (i.e., Triton X-100) are used to increase solubility of non-polar insoluble proteins (Helenius & Simons, 1975). Proteins retaining tertiary and quaternary structures remain soluble in water, since non-polar hydrophobic regions are generally oriented toward the center of the protein or within cell membranes (Tanford, 1962). Thus, reducing agents [e.g., dithiothreitol (DTT)] are used to breakdown disulfide bonds (S-S) between cysteine residues (Cleland, 1964), while sodium dodecyl sulfate (SDS) detergent is added to coat hydrophobic regions of proteins with negative charge and overwhelm positive charges in proteins; this aspect is crucial for resolving proteins in accordance to their molecular mass (discussed below). Reducing agents are required in subsequent sample preparation stages and may interfere with determining the protein content (discussed below), and thus it is recommended

where possible to avoid or minimize addition before quantification.

The detection of certain proteins may require further optimization of buffer components (typical inclusions and concentrations in Table 1). For instance, high concentrations of salts (i.e., NaCl) or detergents to enhance breakdown of organelle and nuclear membranes, such as radio-immunoprecipitation buffer to ensure nuclear disruption (Holden & Horton, 2009). An example of such necessary changes is for the extraction of the DNA-bound proteins in muscle tissue using hyperosmolar lysis buffers to effectively release DNA-bound proteins, which can increase the ability to detect low abundant proteins (Girgis et al., 2014). Therefore, the cellular location and DNA binding of target protein(s) should be carefully considered before determining optimal extraction buffers, which could impact on quantification and therefore conclusions being drawn.

Disruption of cell membranes during homogenization also releases proteases, kinases, and phosphatases, and despite reduced storage temperatures (i.e., 4 °C), protein degradation may still occur due to retained enzymatic activity (Scopes, 1994). Therefore, protease inhibitors that suppress the activity of a variety of proteases must be added either individually or as a commercially dissolvable preparation with the aim of preventing a broad a range of protease activities (e.g., aprotinin for serine and E-64 for cysteine proteases) (Grabski, 2009). Metalloproteinases may be inhibited through the use of metal chelators such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) via chelation (binding of metal ions) of Mg^{2+} and Ca^{2+} which are required for protease activity (Auld, 1995). Further to this, EDTA and EGTA additionally inhibit serine/threonine (Ser/Thr) phosphatase interactions; however, some caution is warranted since EDTA disrupts Na^+ orthovanadate-mediated inhibition of Tyr phosphatases (Huyer et al., 1997). Other components are also required to maintain

protein phosphorylation states, changeable by phosphatases released by homogenization. As an example of the importance of this step, the addition of phosphatase inhibitors was shown to result in increased signal intensity for phosphorylated MAP kinase within neuronal cells, whereas total-MAP kinase intensity remained constant (Sharma & Carew, 2002). Interactions of tyrosine (Tyr) and alkaline phosphatase are generally inhibited through the addition of Na^+ orthovanadate, acting as a competitive Tyr phosphatase inhibitor (Gordon, 1991). Additional inhibitors of Ser/Thr phosphatases such as sodium fluoride and β–glycerophosphate may be used in conjunction with these chelators, increasing the effectiveness of the buffered solution to maintain phosphorylated protein status. Typical buffer components and concentrations can be found in Table 1. It is important to note that any equipment or buffers used within sample processing should be pre-chilled and ideally kept cold on ice.

Cellular subfractionation and immunoprecipitation

Isolating specific cellular fractions (myofibrils, sarcoplasm, mitochondria, collagen) is commonly performed as part of the homogenization process for multiple analysis (e.g., WB and mass spectrometry; Wilkinson et al., 2008) as it may be of interest to determine proteins with specific localizations (e.g., GLUT4 translocation across the plasma membrane; Miura et al., 2001) or transcription factor DNA binding (Girgis et al., 2014); however, successful isolation may require additional buffer components (discussed previously). For skeletal muscle, as a first step, soluble proteins within the sarcoplasm are isolated and separated from insoluble (in standard WB buffers) myofibrillar fractions. The sarcoplasmic fraction may be subject to further organelle separation through differential centrifugation and isolation methods (utilizing fraction-specific buffers) into mitochondrial and cytosolic fractions (Huff-Lonergan et al., 1995; Dimauro et al., 2012). Commonly mitochondrial isolation may involve the passing of the homogenized sample through a Dounce homogenizer as it preserves mitochondrial morphology, minimizing membrane disruption (Dounce et al., 1955). Many of these isolation methods are relatively crude in nature; therefore, steps need to be taken to assess the purity and specificity of preparations (e.g., measuring the presence or absence of fraction-specific proteins). Most protocols require differential centrifugation techniques (Huff-Lonergan et al., 1995; Wilkinson et al., 2008), whereby insoluble myofibrillar fractions (containing mitochondria) are pelleted by higher speed centrifugation (e.g., 11 000 g), with remaining mitochondrial proteins isolated following re-suspension, and a slower speed centrifugation (e.g., 1000 g) before removal of the mitochondrial containing supernatant. While crudely isolating mitochondria, pure separation is difficult as skeletal muscle mitochondria are integrated with structures in the muscle (Rasmussen & Rasmussen, 2000). It should also be noted that there are two main areas within the muscle where mitochondria are associated: subsarcolemma and inter-myofibrillar (IM). Robust separation of each fraction requires additional steps to mechanically or enzymatically (e.g., trypsinization) release IM mitochondria from the myofibrils, which may in turn cause protein degradation to other structures or proteins within the myofibrillar fraction (Rasmussen & Rasmussen, 2000). It is important to note the addition of trypsin will require quenching typically through the addition of albumin, increasing the total protein content in subsequent quantification, and samples should, therefore, be thoroughly washed (Beltran Valls et al., 2014). A recent method has been described, which involves short proteinase treatment and homogenization in ionic buffers followed by two-stage centrifugation. This method showed high mitochondrial integrity and purity following isolation (Rasmussen & Rasmussen, 2000). However, even with specific isolation techniques, the chance of contamination or protein degradation remains high. It is, therefore, crucial to assess the relative purity for fractions isolation by performing a WB for proteins known to be associated with the desired location [e.g., GAPDH and COXIV or cytochrome C for cytosolic and mitochondrial fractions, respectively (Dimauro et al., 2012; Beltran Valls et al., 2014)]. Publications using such techniques should be expected to provide good evidence of fraction(s) purity.

Additional sample processing by immunoprecipitation (IP) may be desirable before SDS-PAGE as it allows the investigation of potential protein–protein interactions (Crossland et al., 2013), and concentration of proteins of low abundance to be extracted for accurate detection by subsequent blotting (Rascón et al., 1992). Initially, the primary antibody (1°Ab) specific to the desired protein is added to the homogenized lysate to form an immune complex that binds to protein A or G beads (generally agarose), which are centrifuged at low speed and the pelleted protein-antibody complex bound beads removed. Proteins may then be eluted and utilized for WB (Huang & Kim, 2013). Through this, physically interacting proteins will be captured by the target 1°Ab, allowing their subsequent denaturation and separation by SDS-PAGE. These interacting additional proteins may then be blotted for, providing valuable insight into potential protein–protein interactions as their detection would only be possible if bound to the originally IP-targeted protein. It is important to note that the 1°Ab may also contaminate the sample as

denatured heavy (50 kDa) and light chains (25 kDa) will be present within the blot (Anton, 2008). If the resulting blot contains either a strong background or the band of interest is obscured by the denatured chains, the use of label conjugated protein A or G (e.g., Protein-A-HRP) may be used as they bind almost exclusively to intact antibodies (i.e., the chosen primary antibody) (Lal et al., 2005)

Protein quantification and gel loading

Following protein extraction, each sample requires the standardization of total protein loading per well. Quantification of protein content may be determined through colorimetric (Bradford, 1976) or UV absorbance (280 nm) (Desjardins et al., 2009) methods. The Bradford assay utilizes a colorimetric change of Coomassie Brilliant Blue G-250 from 465 nm (brown) unbound to 595 nm (blue) when bound to protein (Bradford, 1976), with protein concentration being proportional to the absorption at 595 nm with reference to a standard curve of known concentrations (typically 100–1500 µg/ml). This procedure is simple to perform and requires only basic spectrophotometric equipment; furthermore the reaction is rapid (~2 min) and the bound product is stable for ~1 h at room temperature (Bradford, 1976). A number of other methods based along similar biochemical colorimetric properties such as the Lowry, bicinchoninic acid, and ortho-phthaladehyde assays are also regularly reported for protein concentration measures in WB procedures (Noble et al., 2007). While popular, colorimetric assays have a number of potential disadvantages in that they require more sample than other modern methods (i.e., UV absorbance) and are susceptible to pipetting errors of either the samples or during standard curve construction, along with suffering from interference by a number of buffer/tissue components [e.g., β-mercaptoethanol (β-MCE)] or hemoglobin (Doumas et al., 1981). Measuring protein content through UV absorption with the use of micro-spectrophotometric (e.g., Nanodrop) equipment may be more suitable due to the comparatively small sample volume (0.5–2 µl), wide quantification range (0.1–3000 µg at 280 nm), and potential for complete sample recovery once the measurement is complete (Desjardins et al., 2009). This procedure utilizes the absorbance of UV light at 280 nm by amino acids containing aromatic rings (i.e., phenylalanine, tyrosine, and tryptophan) allowing accurate quantification (Layne, 1957). Erroneous measurements may occur if the sample comprises both insoluble and soluble proteins that will affect absorbance measurements, demonstrating the importance of sample homogeneity. Moreover, to ensure reproducibility, standard curves of known protein concentrations should be performed with each batch to validate the approach. Once quantified, aliquoted samples may be diluted to the desired concentration with the addition of appropriate buffers, in preparation for sample loading.

The final step in processing samples requires the denaturing (unfolding) of secondary/tertiary structures in proteins, allowing separation based on the primary amino acid sequence theoretically in accordance to the predicted molecular weight. To do this, standard concentrations of samples (acquired as above) are mixed with Laemmli buffer (Laemmli, 1970), the composition of which serves a number of important functions. Thiol containing cysteine residues form disulfide bonds, which govern protein folding and stabilize the secondary/tertiary structure of proteins (Creighton, 1988). The addition of a reducing agent, typically β-MCE (DTT or TCEP (tris(2-carboxyethyl)phosphine) may also be used) cleaves disulfide bonds destabilizing the secondary and tertiary structure, unfolding the protein (Anfinsen, 1973). Due to the volatility of β-MCE, its addition should occur immediately before use. Furthermore, high concentrations of β-MCE or other denaturing agents (i.e., DTT) can interfere with protein assays and therefore should be added post protein quantification (Krieg et al., 2005). Finally, to allow separation through the application of an electrical current, all protein R-groups (functional amino acid groups) are coated with negative charges through addition of SDS. As SDS binds to the primary structure of proteins (1.4 g per 1 g of protein), the overall charge of the protein becomes relative to its molecular weight, and it is this that forms the basis of the established separation of proteins through a polyacrylamide gel matrix (Smith, 1984).

Typically 10–100 µg of total cellular protein per lane is loaded (Taylor & Posch, 2014); however, this is generally within precast gels and will be determined by multiple factors. These will include the thickness of the gel, and the lane width, requiring a greater volume of sample. Nonetheless, the final concentration of protein to detect a given antigen should be determined by the end user in accordance to detection efficacy (i.e., via running titrations of protein). Although it is highly dependent on the protein of interest, it is common for protein concentrations of human muscle biopsies to be adjusted to 1–2 µg/µl, with a total of 15–30 µg protein per lane being typically loaded (Franchi et al., 2014). Proteins of lower abundance within the sample may need increased quantity to be loaded; for example, the vitamin D receptor (VDR) is highly expressed within the kidney, whereas within skeletal muscle, it is relatively low requiring a greater amount to be loaded (i.e., ~60 µg; Girgis et al., 2014). This can be achieved by concentrating the sample (i.e.,

evaporation or protein precipitation) or utilizing gels with larger sample wells.

In order to have good resolution and identification of the band(s) of interest, proteins are separated by their mass (in accordance with their primary structure/AA sequence), as such it is essential to include protein standards containing a mixture of predefined proteins of known molecular weight markers to confirm the band of interest is the correct mass (Weber & Osborn, 1969). Such standards are generally loaded into the first and last lane of a gel. The choice of standard will be dependent on the resolution required near a molecular-weight region, along with potential analysis requirements, such as confirmation of the size of protein targets. Standards may already be pre-stained for ease of visualization of separation and confirmation of an effective transfer onto membranes. Standards may also provide confirmation of either 1°Ab or 2°Ab binding as they can contain known binding sites for these targets and therefore should produce detectable bands, e.g., a purified protein of interest. The inclusion of such internal standards allows the optimization of subsequent processes through confirmation of antibody binding (Mahmood & Yang, 2012). Therefore, SDS-PAGE systems may be tailored to ensure optimal separation and resolution of the desired protein targets depending on their molecular weight. Further to this, the inclusion of specific molecular weight standards may be used in conjunction with additional methods of confirmation of sufficient separation (e.g., use of stain-free gels or membrane staining, discussed below).

Polyacrylamide gel electrophoresis (PAGE)

Following these sample preparation steps, PAGE is performed to separate out denatured and negatively charged proteins based on their molecular weight. Separation of protein samples within polyacrylamide gels occurs due to the frictional resistance of a protein as it migrates through pores formed between polymer chains within the gel (Ornstein, 1964). Polyacrylamide gels comprise polymerized acrylamide monomers along with cross-linking N,N′-Methylenebisacrylamide monomers (Raymond & Weintraub, 1959), creating uniformly sized pores, dependent on both monomer concentration and cross-linker ratio. As an electrical current is passed, proteins move through pores within the gel structure; as such, altering the bis-acrylamide concentration regulates pore size and consequently the ability of larger proteins to migrate. Gels with a higher acrylamide concentration (e.g., 20%) impede the movement of larger proteins to a greater degree than those of a smaller molecular weight but better resolve those of lower molecular weights (e.g., 4EBP1 ~20 kDa) (Chrambach & Rodbard, 1971). Similarly, if the desired target is a large protein (e.g., mTOR ~289 kDa), a lower concentration gel (e.g., 7.5%) may be required for optimal resolution. Alternatively gradient gels (e.g., 4–12%) provide uniform resolution across the molecular-weight spectrum (Rath et al., 2013). Other gel types such as agarose may be used, but are less common as they are predominantly used for very large molecular-weight proteins (e.g., titin isoforms 700–4200 kDa) giving superior separation when compared to polyacrylamide gels (Warren et al., 2003). Agarose gels may be hand-cast but require storage at 4°C to prevent drying out. Other hand-cast gels may also require use soon after casting and may vary between runs due to the short shelf life of some chemicals. The choice between commercial and hand-cast gels is generally the preference of the user, but commercial gels are generally more consistent. Ultimately, the concentration of the cross-linking molecules and the molecular weight of the protein(s) of interest are the determining factors for gel choice as taken together these will allow for efficient migration and optimal band resolution. The choice of gel concentration and composition is mainly determined by the molecular weight of the protein(s) of interest, as it will allow efficient migration and optimal band resolution.

Whereas electrophoresis of nucleic acids utilizes a constant pH within the buffer and gel to achieve discernable separation (Westermeier, 2005), protein samples require a discontinuous buffer system (Ornstein, 1964). Discontinuous systems utilize gels separated into two regions, comprising a "stacking gel" above a "resolving or separating gel" with larger and smaller pores, respectively. Discontinuous systems are designed to focus protein samples and allow clear resolution of proteins. Initially, proteins migrate quickly through the large pore stacking gel until they reach the resolving gel, whereupon the smaller pore size slows migration, causing the proteins to stack together into compact bands (Ornstein, 1964). The second principle utilizes the electrophoretic migration of both ions and proteins through a pH-buffered solution. Chloride ions present within the gel have a higher mobility and therefore migrate faster than denatured proteins, establishing a leading ion boundary (Ornstein, 1964). Within traditional Tris-HCl stacking gels (pH 6.8), a trailing boundary of glycinate ions form behind migrating proteins due to reduced mobility at a low pH (Walker, 1994). Ultimately, migrating proteins are sandwiched between the two ion boundaries, stacking the sample into tightly focused bands, whereupon they migrate into the resolving gel containing smaller pores, slowing the progression of proteins dependent on their size as previously mentioned. Tris-HCl resolving gels are typically formed at pH 8.8; this higher pH allows the ionization of the trailing glycinate, increasing its

mobility (Ornstein, 1964). Consequently, both chloride and glycinate boundaries will migrate past the protein samples, no longer constricting them into focused bands, allowing the unimpeded separation of proteins. Alternative gels and discontinuous buffer systems are available that may be better suited to the resolution of either larger or smaller molecular-weight proteins depending on sample composition. For example, Bis-Tris systems utilize different buffers containing either 3-(N-morpholino)propanesulfonic acid (MOPS) or 2-(N-morpholino) ethanesulfonic acid (MES), which function as trailing boundary ions instead of glycinate (Hachmann & Amshey, 2005). These allow greater resolution of mid-sized (~75 kDa) or smaller (<36 kDa) proteins with MOPS or MES, respectively. Bis-Tris gels are cast at pH 6.8, offering significantly longer shelf life compared with Tris-HCl gels as they do not undergo acrylamide hydrolysis due to their acidic nature. Generally, electrophoresis is undertaken using a constant voltage, rather than a constant current, due to the linear relation of protein migration and voltage. As current is dependent on the voltage and resistance, a constant current will not control protein migration as changes in resistance (i.e., warming of the buffer) will cause the voltage to fluctuate. Depending on the apparatus, electrophoresis is typically performed for 60 min with a constant voltage of 200 V to give a suitable separation of protein lysates; however, less time may be required for smaller molecular-weight proteins.

Electrotransfer

Following the separation, the proteins are electrophoretically transferred to a membrane (electroblotting; with high affinity for protein), thereby immobilizing the separated proteins, allowing subsequent probing with antibodies, and providing additional durability compared to gels (Towbin et al., 1979). Utilizing the same principle as PAGE, the negatively charged proteins in the gel are transferred across onto the membrane when a lateral electric current is applied while immersed in a buffered solution (termed wet transfer). The membrane is placed directly upon the gel ensuring a mirror image transfer of proteins occurs. Proteins are typically transferred onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes; originally nitrocellulose membranes were used for their immediate binding and immobilization of proteins (Towbin et al., 1979; Burnette, 1981). As nitrocellulose membranes are not hydrophobic, they are easily hydrated for use within wet or semi-dry transfers. However, nitrocellulose membranes can be fragile, making them incompatible with the current commonplace practices of stripping and re-probing for alternative

proteins (discussed later in stripping and re-probing). The development of more durable hydrophobic PVDF membranes allows the possibility to strip and re-probe, as they are more chemically inert and robust (Kurien & Scofield, 2006). PVDF membranes bind proteins through hydrophobic interactions (MacPhee, 2010) and are capable of binding greater amounts of protein (~150 $\mu g/cm^2$) than other substrates (Matsudaira, 1987). Although the use of PVDF membranes may be advantageous in capturing greater amounts of protein, membrane–antibody interactions are more likely to occur, generating higher backgrounds when exposing the blots, consequently increasing the importance of performing thorough wash steps (Mahmood & Yang, 2012). The hydrophobic nature of PVDF membranes requires an initial pre-soaking in methanol to allow the infiltration of the buffer and the binding of proteins (Mansfield, 1995). Another variable to consider is the choice of pore size (i.e., 0.45–0.025 μm) of the membrane, as this will affect the binding of larger or smaller proteins (Burnette, 1981; Tovey & Baldo, 1987). Smaller proteins, such as cytochrome C (12.5 kDa), have been shown to have a reduced binding capacity upon membranes with a pore size of 0.45 μm (Burnette, 1981). Thus, to probe for smaller molecular weight proteins (<20 kDa), a smaller pore size membrane is advisable as this will bind larger quantities of protein. It is important to note that protein transfer onto PVDF membranes may be inhibited by high SDS concentrations (Mozdzanowski et al., 1992), so prior to transfer gels should be thoroughly washed in distilled water (e.g., 2 min for thin gels), and then equilibrated in transfer buffer (e.g., 5 min) to remove excess SDS.

Methods of transfer are numerous. Traditional wet transfers involve successive layers of a cassette backing, fiber pads, blotting paper, the polyacrylamide gel, the chosen membrane, blotting paper, and the cassette front being "sandwiched" together and submerged between an anode and cathode within a transfer tank (Towbin et al., 1979; Burnette, 1981). The transfer process as described by Burnette (1981) was achieved over 22 h; however, this duration is dependent on the size of the protein(s) of interest. Insufficient transfer time will result in weak signal or no intensity, as significant quantities of proteins will remain within the gel. Conversely, excessive transfer time will generate a poor signal as proteins pass through both gel and membrane; however, PVDF membranes generally have smaller pores compared with nitrocellulose, reducing the amount of protein passing through. As with electrophoresis, the rate of protein movement is dependent on its molecular weight, and thus smaller proteins may require less time for transfer. Gel staining allows the evaluation of transfer efficiency, through visualization of

protein remaining within a gel (e.g., using Coomassie/silver/Ponceau stains or stain-free gels), removing some aspects of transfer uncertainty (Colella et al., 2012). Other transfer techniques such as semi-dry transfers are frequently utilized as they can offer faster transfer times (reduced to just over an hour), depending on individual requirements or time constraints (Kurien et al., 2015). Most of the conventional commercial systems are easy to use, reliable for transfer of proteins of a wide range of sizes, and use relatively inexpensive reagents. Still faster transfers may be attainable using recently developed systems such as the Trans-Blot® Turbo™ Transfer System, but these may not work well with some proteins (i.e., <100 kDa) and require more expensive consumables.

The composition of the transfer buffer will also dictate the efficiency of transfer, especially for proteins of a high molecular weight. Generally, wet transfer solutions are buffered to pH 8.3 and contain Tris-base along with glycine (Towbin et al., 1979). It is important to note that the buffer pH should not be adjusted through the addition of acidic or basic solutions, as this will result in higher conductivity through greater ion content, thus increasing the temperature of the solution and potential background interference and/or inefficient transfer. These buffers may be pre-chilled to prevent overheating, due to the high current, and the deformation of the membrane, with transfer tanks commonly containing ice blocks. Methanol was used within the original transfer buffers (Towbin et al., 1979; Burnette, 1981) as it increases the ability of membranes to bind proteins and prevents gel swelling. However, since the inclusion of methanol within these buffers may decrease the transfer of larger molecular-weight proteins (>100 kDa) as it reduces the pore size of within gels (MacPhee, 2010), it may be advisable to exclude methanol when probing for larger proteins. For the majority of proteins analyzed via WB techniques, low-ionic strength buffers and low electrical currents are optimal (Alegria-Schaffer et al., 2009). If these generic rules are followed, then successful transfer is highly likely. Reversible staining of the membrane and/or gel with Ponceau, e.g., will reveal protein loading, commercial systems for reversible staining also exist and have been extensively validated for this purpose (Antharavally et al., 2004; Alegria-Schaffer et al., 2009). Unequal loading will be displayed as noticeable differences in multiple protein band(s)/lane(s) intensities, in this instance the variability may be due to unequal sample protein quantities caused by different sample concentrations, or mistakes in the sample volume loaded into individual lanes. Protein lysate concentrations should be reanalyzed to determine if it was the former. Without efficient and complete transfer of proteins, accurate quantitation and analysis will be compromised.

Blocking

Although one of the simplest technical steps to perform in the WB process, blocking is important as it can prevent non-specific binding of antibodies (1°Ab and/or 2°Ab) to the membrane (Jensen, 2012). As membranes have a high affinity for binding proteins and therefore antibodies, blocking reduces background in subsequent steps. Different solutions may be used for this stage of the protocol, each having their own benefits and limitations. Non-fat-dried milk diluted in Tris Buffer Saline Tween-20 (TBST) is often used and is cheap and widely available, and milk proteins are not, however, compatible with all antibodies. For example, it has been reported that bovine serum albumin (BSA; normally 5%) should be preferentially used for biotin and anti-phospho-protein antibodies as milk contains casein, which is a phospho-protein, and biotin, rendering it likely to interfere with the WB as the phospho-specific antibody may cross-react with the casein present in the milk (Mahmood & Yang, 2012). Awareness of this issue is ever more relevant with the continued development of numerous specific, non-cross-reactive anti-phosphoamino acid antibodies. Although these antibodies greatly enhance phosphoamino acid-specific analysis and negate the need for radioactive reagents, i.e., ^{32}P, high backgrounds have been observed when these antibodies are used during immunoblotting (Michalewski et al., 1999). Possible explanations for this technical challenge include the existence of phosphorylated proteins in various blocking solutions (as alluded to above) or inappropriate membrane-blocking conditions. Work by Michalewski et al. reported that the binding of anti-phosphoamino acid antibodies to proteins and membranes critically depended on blocking conditions, with a combination of amicase (5%), BSA (5%), and membrane-blocking agent (5%) to be most effective in reducing non-specific binding (Michalewski et al., 1999).

For the preparation of both milk and BSA, a 2.5–5% weight: volume solution is most commonly used (Crossland et al., 2013; Franchi et al., 2014), in TBST or phosphate-buffered saline (PBS). The mixing must be thorough and the solution should then be filtered to prevent grains contaminating the blot during development. A further (albeit less common) option for blocking is using highly purified non-animal proteins (e.g., isolated casein), although these are much less cost-effective. Appropriate guidance on blocking conditions is usually provided by commercial companies alongside the antibody-specific information; however, because not all blocking

conditions are suitable for all target proteins, validation and testing for each individual protein of interest is always recommended (Spinola & Cannon, 1985). Whichever approach is chosen an optimal blocking, buffer should improve the sensitivity of the WB by reducing background and improving the signal-to-noise ratio (measured as the signal obtained in a sample containing the target analyte vs that obtained with a sample without the target analyte). Ideally, a blocking agent will bind to all sites of non-specific interaction, eliminating all background without altering access/interaction to the protein(s) of interest for antibody binding. The choice of blocking agent should be based on the antigen itself and the type of 2°Ab conjugation (see below). For example, in assays where alkaline phosphatase conjugates are used, TBST should be selected, as PBS will interfere with these phosphatases due to the presence of sodium phosphate (and in some solutions potassium phosphate also). If PBS is to be used for intermediate steps, the membrane should be sufficiently washed in TBST to remove excess sodium phosphate before addition of the substrate.

It is not only the choice of blocking agent that can affect WB outcomes; volume of blocking agent and indeed the incubation period for blocking (which may vary from an hour to overnight; Gershoni & Palade, 1983) are also important factors. Too little blocking agent (low concentration) or too short an incubation period will increase the potential for non-specific binding of the 1°Ab to the membrane-bound proteins which could result in excessive background and/or reduced signal-to-noise ratio. Conversely, an incubation period that is too long and/or uses excessive blocking agent may interrupt antigen-antibody interactions, also causing a reduction in signal-to-noise ratio (Mahmood & Yang, 2012). In a comparative study assessing the efficacy of commonly used blocking protocols to minimize non-specific background and promote immunoreactivity of antibodies (monoclonal, polyclonal or biotin-conjugated) against a phospho-amino acids, the authors recommended the use of a solution composed of 5% BSA, 5% Amicase® (Sigma; a mixture of free amino acids with virtually no unhydrolyzed peptides and minimal inorganic components), and 5% membrane-blocking agent in Tris-buffered saline containing 0.05% Tween 20 [to aid in the removal of residual SDS from earlier steps (Zampieri et al., 2000)] for 45 min at room temperature to achieve good-quality low background WB (Michalewski et al., 1999). As with most common blocking agents (Gershoni & Palade, 1983), incubation for longer than 1.5 h led to reduced signal strength (signal to noise), with 30 min or less resulting in unacceptably high backgrounds. Therefore, in our experience, initial optimization is undertaken using a 5% milk solution in TBST for

1 h, whereupon the background levels are assessed and concentrations/blocking agent subsequently altered. No single blocking agent is ideal for every WB assay as each antigen-antibody pairing has unique characteristics. Consequently, the chosen blocking strategy must be optimized for each individual application, with the above considerations being kept in mind.

Primary antibodies and determining specificity

The principle of the WB is the detection of protein(s) through the binding and recognition of antibodies (Ab) to one or more targets; this interaction should be highly specific between a portion of the antigen (protein) or epitope and the specific recognition sites found on the fragment antigen-binding (Fab) region of the antibody termed a paratope (Kurien et al., 2011) (Fig. 1). The 1°Ab should be thoroughly assessed and validated to be specific and sensitive enough to detect the intended target protein. It is important to check that the antibody is specific toward the native or denatured protein, as the denaturing treatment of protein samples prior to SDS-PAGE may alter the exposure and availability of the epitope, affecting antibody binding affinity. In some cases, it may be necessary to use "native-specific" monoclonal antibodies (Tino et al., 2000). The targeted peptide sequence may be available from the supplier to allow confirmation of specificity and region of binding; however, occasionally this may be unavailable proprietary information. Traditionally 1°Ab are produced through immunization of the host using purified target proteins, whereas modern approaches utilize synthetic peptides, often producing Ab toward short denatured 8–10 amino acid sequences. Isolation and purification is generally achieved through affinity chromatography isolating antibodies and small proteins and/or anion-exchange filtration depending on the class (i.e., IgG, IgM) (Clezardin et al., 1986). Predicted and confirmed species cross-reactivity information is often only available through the vendor; however, binding will entirely depend on the antigen region. When choosing a 1°Ab, there may be multiple forms available from different vendors, ideally each would bind to a unique antigen upon the protein of interest, allowing accurate assessment of the protein's abundance. However, this is often not the case, and assessment of the previous literature utilizing that antibody is strongly advised. Some proteins may be orthologous and contain the same or similar sequence to other species. Thus, if a 1°Ab is specific to an epitope with this sequence, it may be used to probe other species (i.e., GAPDH 1°Ab may be used on human, rat, and mouse tissue). Depending on the protein of interest, extensive testing of multiple antibodies may have

already been undertaken, allowing the most suitable antibody to be selected. For example, the VDR has low expression within skeletal muscle, and in order to find a 1°Ab capable of detecting it by WB and immunofluorescence, extensive validation of a panel of multiple 1°Abs was required (Wang et al., 2010). The identification of a highly specific VDR 1°Ab (D-6; Santa Cruz Biotechnology, Cambridge, UK) was confirmed and is believed to be the most representative. Assessment of new antibodies for a target antigen requires even more careful testing, including the use of a variety of appropriate positive and negative controls (discussed below).

Specificity and performance of the 1°Ab antibody is also dependent on whether it is monoclonal (mAb) or polyclonal (pAb). Both have disadvantage/advantage; pAb are produced from differing B-cell lineages, recognizing multiple epitope regions on an antigen. They are generally more cost-effective (Lipman et al., 2005) and provide more antibody molecules that can target the protein of interest, producing potentially a greater level of sensitivity upon analysis (MacPhee, 2010). However, their specificity can also be compromised, due to greater possibility of non-specific binding (MacPhee, 2010). In contrast, mAb provide highly consistent and specific binding to a specific and known epitope on an antigen, as they are produced from a single cell lineage, raised against a single specific epitope (Lipman et al., 2005; MacPhee, 2010). Yet binding affinities of mAb can suffer if the epitope structure is affected in any way through denaturing or electrophoresis for example (Lipman et al., 2005).

Depending on the primary amino acid sequence of the target protein, similar epitopes may be present within degradation products or alternate isoforms, potentially presenting additional bands. Degradation products will migrate ahead of the band of interest, due to the decreased molecular weight. 1°Ab affinities toward alternative isoforms may not interfere with data interpretation if the bands are sufficiently separated (i.e., have different molecular weights). For example, certain antibodies toward P70 S6K1 (70 kDa), a critical protein in the mRNA translational initiation pathway and one of the most probed of all in the muscle and exercise field, may bind to the isoform P80 S6K (80 kDa). P80 S6K encodes a nuclear localization signal and contains an additional 23 amino acids and would be present above P70 S6K1 when blotted (Thomas, 1993). Nonetheless, sufficient electrophoretic separation between the two isoforms and appropriate controls may allow correct identification. For example, insulin-treated L6 myotubes increased P70 S6K1 phosphorylation (Somwar et al., 1998) but not P85 S6K allowing, in this instance, identification of the correct band; nonetheless, this does not guarantee specificity.

However, the assessment of a bands molecular weight may not always be suitable, as some proteins may migrate to a non-predictable region. For instance, the mTOR regulator REDD1 has a predicted molecular weight of 25 kDa; however, it is detectable at 35 kDa due to multiple lysine residues (increased positively charged residues) (Chang et al., 2009). This highlights the importance of knowing the migrating properties of the target protein, and if unexpected bands occur, literature investigation may be required.

In order to validate the specificity of a new 1°Ab, it should be tested against a positive lysate or purified protein control, giving a detectable band at the correct molecular weight and a negative sample from a tissue known not to express the intended target [The Human Atlas provides reliable protein expression data (http://www.proteinatlas.org)], resulting in no detectable band. Sometimes, it may be appropriate to include a specific knock-in/out (e.g., via shRNA or siRNA) sample to allow confirmation of a target within the same tissue type. For example, overexpression of AKT isoforms (an essential signaling protein for muscle hypertrophy/atrophy) within rat skeletal muscle following shRNA produced detectable bands at the predicted molecular weight (~40 kDa) compared with control samples (Cleasby et al., 2007). Conversely, knockdown of AKT, again within skeletal muscle, demonstrated a reduction in band intensity at the same molecular weight compared with control samples. Similarly protein inhibitors (e.g., LY294002, rapamycin, etc.) known to block specific phosphorylation pathways can be used. For example, the addition of LY294002 to cultured cells inhibits PI(3)K, resulting in decreased phosphorylation of down-stream intermediates (i.e., AKT/P70 S6K1) (Rommel et al., 2001). Thus, if probing for phosphorylated P70 S6K1, cultured L6 cells treated with/without insulin and LY294002 will provide a robust positive (greater band intensity) and negative control (reduced intensity), respectively, compared with untreated samples. In this instance, the inclusion of an untreated sample alongside an inhibited negative control will confirm the reduction in band intensity is in fact due to decreased expression, rather than a loss of detection. Despite rigorous testing of antibodies with appropriate positive/negative controls, additional bands may still be present or insufficiently separated making identification and quantitation of the correct band (if present) unreliable. Absolute confirmation of the presence of a protein in a given band may be achieved by mass spectrometry via determination of the peptide sequence (Trauger et al., 2002). Briefly, the band of interest is excised and the mixture of proteins digested by trypsin into small peptide sequences capable of being sequenced by liquid chromatography–

mass spectrometry (LC-MS/MS). Utilizing this approach, however, requires access to highly specific and costly equipment and technical expertise, but can provide validation of antibody specificity. Ultimately, positive and negative controls will help establish the degree of non-specific binding and potential false-positive bands, along with the confirmation of increased/decreased protein expression, giving confidence that the highlighted band is indeed the correct one.

Post-translational modifications

Crucial for determining the relative importance of potential signaling mechanisms is the ability to detect PTMs such as the alterations in reversible phosphorylation states of various proteins (phosphoproteins). The phosphorylation of a protein will alter multiple aspects of its interactions, including localization, conformational shape, hydrophobicity, and activity (Polyansky & Zagrovic, 2012). Phosphospecific-antibodies capable of distinguishing between short epitopes containing phosphorylated or unphosphorylated amino acid residues, typically Thr, Ser, and Tyr have been developed (Nairn et al., 1982). Proteins may also be phosphorylated at multiple loci through different signaling mechanisms, and thus it is important to choose the correct phosphorylation site, depending on the pathway or response of interest. For example, the activity of eukaryotic translation initiation factor 4E (eIF4E), a subunit of eIF4F (eukaryotic translation initiation factor 4F), crucial for the initiation of protein synthesis is regulated through the phosphorylation of 4E-BP1 (eIF4E binding protein 1). 4E-BP1 is phosphorylated by FRAP/MTOR at Thr37/46, but requires additional phosphorylation at Ser65 and Thr70 to disassociate from eiF4E (Gingras et al., 1999). Simply measuring changes in Thr37/46 phosphorylation may be inadvertently misleading, as additional phosphorylation may be required for activation (Gingras et al., 1999). In this case, both phosphorylation sites (i.e., 37/46 and 65/70) should be probed for when investigating changes in potential signaling mechanisms of protein synthesis.

A myriad of other PTMs have important roles on various physiological states including but not limited to ubiquitination (Paul et al., 2012), glycosylation (Péré-Brissaud et al., 2015), and methylation (Voelkel et al., 2013), and changes may be assessed via WB. For example, starvation-induced skeletal muscle atrophy and protein degradation are associated with an increase in ubiquitinylation of proteins (Paul et al., 2012). This may be assessed using a 1°Ab targeting ubiquitin that will bind to multiple ubiquitinated proteins throughout a blot. Thus, in samples from starved or atrophying muscle, the band intensity of the lane will be increased compared with control samples, demonstrating increased protein ubiquitination, suggestive of increased proteolysis. This assessment of ubiquitination needs to be accompanied by separate measures of proteins involved within the proteolytic process, such as FoxO3 and AKT, confirming (or otherwise) increases and decreases in phosphorylation levels in atrophic muscle samples (Zhao et al., 2007). This same principle of detecting the incorporation of a label onto multiple proteins permits the monitoring of the translation process, e.g., using puromycin in cell culture or preclinical models (Schmidt et al., 2009). Here, puromycin, a structural analog of aminoacyl tRNAs, is incorporated into newly synthesized proteins, which are subsequently probed using an anti-puromycin 1°Ab. Measuring lane intensity provides a semi-quantitative assessment of global protein synthesis (Schmidt et al., 2009). As previously mentioned, known signaling intermediates (i.e., AKT/P70 S6K1) need to be assessed to ensure that changes to puromycin incorporation are matched to increased anabolic signaling (Crossland et al., 2013). Ultimately, a 1°Ab may be specific for a single phosphorylated protein or for a specific PTM component such as ubiquitin that may be present within multiple proteins. Thus, even within the same sample, assessments of signaling cascades (phosphorylation), protein degradation (ubiquitination), or protein synthesis (puromycin incorporation) may be made, allowing a comprehensive investigation of protein metabolism. Further PTMs may be assessed by WB such as glycosylation of a protein, which has a substantial impact upon multiple aspects, including protein folding, conformational changes, and stability along with solubility (Rudd & Dwek, 1997). The two major forms of glycosylation are serine/threonine O-linked or asparagine N-linked carbohydrates. This will increase the molecular weight of the glycoprotein, and the degree of glycosylation can be assessed by initially treating samples with a endoglycosidase (e.g., PNGase) to remove N-linked glycans. As a result of removing the carbohydrate moieties, additional bands will be detected at a lower molecular weight (Péré-Brissaud et al., 2015).

Secondary antibodies

Secondary antibodies (2°Ab) are required for the indirect detection of a target antigen bound by a 1°Ab. Typically 1°Ab will not be conjugated to a label for detection, resulting in the need for 2°Ab (conjugated to a reporter function, e.g., horseradish peroxidase [HRP], specific fluorophores) capable of binding to the fragment crystallizable (Fc) region on a 1°Ab, allowing subsequent detection by a camera or imaging device (discussed below). The crucial aspect of utilizing a 2°Ab is the ability to amplify the

detectable signal since multiple 2°Ab can bind to a single 1°Ab, thus amplifying the detection of low abundant proteins. The choice of 2°Ab will initially depend on the 1°Ab isotype and which animal it was raised within (Lipman et al., 2005). Variations within Ab heavy chain formations will determine their function and class; antibodies from mammals will be one of five classes, IgM, IgD, IgE, IgA, or IgG, with the latter being subdivided into multiple subclasses within rats, mice, and humans (i.e., IgG_{2a}, IgG_{2b}) (Lipman et al., 2005). Primary Ab raised within mice or rats may require isotype-specific 2°Ab; however, rabbits produce only a single isotype of IgG, allowing broad specificity 2°Ab to be utilized (Manning et al., 2012). For example, an IgG 1°Ab raised within a rabbit simply requires an anti-rabbit IgG, whereas a mouse IgG_{2a} would require a 2°Ab specific for the IgG_{2a} isotype. As rabbit 1°Abs suitable for WB purposes are common, this means a single anti-rabbit IgG 2°Ab may serve for multiple targets. However, the use of 1°Ab from different species allows the unique potential for multiplex detection using 2°Ab conjugated to different fluorescent wavelengths for multiple detections upon the same blot (discussed below) (Gingrich et al., 2000). For example, a rabbit IgG 1°Ab specific toward AKT (60 kDa) may be probed for along side a mouse IgG_{2a} 1°Ab for P70 S6K1 (70 kDa) using a red fluorescent anti-rabbit IgG (594 nm) and green fluorescent anti-mouse IgG_{2a} (488 nm). An additional key aspect is the ability to simultaneously probe for both total and phosphorylated expression levels (e.g., pan-AKT vs AKT Ser473) providing 1°Ab are raised in differing species and the epitope regions do not overlap (Georgopoulos et al., 2010). The ratio between these measures gives an indication of a proteins capacity for signal transduction (total) vs activation (phosphorylation), as discussed below. Ultimately, the use of different species-specific fluorescent 2°Abs in a single incubation potentially allows the measurement of two or more targets (i.e., total vs phosphorylated or targets of a similar molecular weight) within the same blot.

Although 2°Ab are raised toward specific epitopes upon the 1°Ab, cross-reactivity may occur with other separated proteins or indeed those used for blocking, due to similar peptide sequences (Ramlau, 1987). Therefore, negative controls should also be undertaken by omitting the 1°Ab incubation to ensure the observed bands are not the result of non-specific binding of 2°Ab. The optimal dilution of the 2°Ab will depend on the expression level of the target protein and the choice of 1°Ab (e.g., GAPDH is highly expressed, requiring higher dilutions to prevent signal saturation); however, the manufacturer's recommendation is often a good starting point. A dilution curve may then used to optimize the concentration needed for the targets being investigated. The 2°Ab solutions are typically made up within the same buffer and blocking solutions as outlined previously, but may require optimization if a high background occurs. Unlike conditions for 1°Ab, secondary incubations are generally undertaken at room temperature for a shorter amount of time, typically 1 h. Occasionally, longer incubations may be required; however, this can lead to increased non-specific binding within the membrane, generating a higher background. For ease of use, in our experience, altering antibody concentrations for different targets, while maintaining incubation times, is preferable.

Detection

The general principles of detection for WB are the same as for other antibody-based assays, such as the enzyme-linked immunosorbent assay (ELISA) (Voller et al., 1978). In general terms, the 2°Ab is conjugated with a labeled compound (i.e., radio-isotope or fluorophore) or enzyme that permits subsequent detection. Historically, this was a radioactive isotope or enzyme exposed against X-ray film (Miura et al., 2001) but now is normally an enzyme or a fluorophore detected by camera (Crossland et al., 2013). The two most commonly employed enzymes are alkaline phosphatase (AP) (Bronstein et al., 1989) and HRP (Kricka, 1991). Both AP and HRP can be used for colorimetric or chemiluminescent detection. When employing HRP, it is important to remember that sodium azide inhibits this enzyme so solutions like those used for blotting and detection should not contain sodium azide, or membranes should be sufficiently washed before exposure. In colorimetric detection (Babson et al., 1966), a substrate (e.g., 3,3'-diaminobenzidine) of HRP is oxidized producing a brown insoluble product. Quantification can then be achieved by scanning the blot with either a dedicated imager or traditional office scanner (although the latter is not recommended, as they are not designed to have a large linear range) and analyzing band intensity using freely available software such as NIH Image J (http://imagej.nih.gov/ij/). A key disadvantage of this form of detection is that the chemical reaction must be stopped, and therefore, optimal reaction conditions need to be determined (reaction time, temperature, etc.) prior to quantitation.

With chemiluminescent detection (Kricka, 1991), HRP luminol is oxidized in the presence of hydrogen peroxide producing 3-aminophthalate, which emits light at 425 nm. A chemiluminescent blot is optimally imaged after 3–5 min of incubation with the substrate and may produce a signal for several hours (Alegria-Schaffer et al., 2009). A key advantage of this form of detection is that the blot can be repeatedly rinsed and exposed to substrate and luciferase to allow multiple exposures, which is useful for

optimization of detection parameters, thereby ensuring the blot is not underexposed or overexposed. However, a potential disadvantage is that the blot must either be exposed to film, which must then be developed, or be placed in a specialized detection system (e.g., Chemidoc, Odyssey™) with the latter being the most common and accurate approach. Both options for visualization of the blot, however, require more expense and equipment than for colorimetric detection. Quantification of the blot will be dependent on the light detection method. If photosensitive film was exposed and developed, subsequent scanning and Image J are commonly employed. More commonly, a specialized detection system, typically utilizing proprietary software, employing densitometry analysis will be used. The proper storage and age of buffers, substrates, stop solutions, and luciferase solutions must similarly be considered as degradation and/or contamination of any one of these can affect each of the steps and potentially prevent detection and/or result in very high background signals. It is also important that the 2°Ab has been properly stored (−80 °C, −20 °C, 4 °C) and is reasonably fresh, as the enzymes will degrade with time. If stored correctly, antibodies may still be viable for multiple years (Argentieri et al., 2013); however, suppliers only recommend storage for approximately a year as extended storage will decrease its effectiveness.

These aforementioned limitations can be overcome using fluorescent detection where the 2°Ab is conjugated to a fluorophore. For this type of detection, no further chemical reactions are required, and the blot can be visualized after exposure to 2°Ab and rinsing. However, it does require further specialized imaging systems. Fluorescent-based imaging has a greater upper linear range of detection (250–500 pg) compared to chemiluminescent (125 pg) (Gingrich et al., 2000). Fluorescent antibodies also have a similar lower range when compared to chemiluminescent detection and so may be more appropriate when investigating higher abundance proteins. A further benefit of fluorescent antibodies is the ability to utilize 1°Ab from different species and subsequent different wavelength 2°Ab for each to perform multiplexed exposures upon the same blot (discussed previously) (Gingrich et al., 2000). This crucial difference can allow the probing of multiple targets with similar molecular weights (e.g., AKT and P70 S6K1) or PTMs (e.g., pan-AKT vs AKT Ser473) using different conjugated fluorophores, specific to different excitation channels (e.g., 594 nm vs 488 nm) (Georgopoulos et al., 2010). When it is desirable to improve the sensitivity for the detection of low abundance target proteins, a couple of approaches may be employed. First, switching detection systems may help as fluorometric detection is

generally similar to chemiluminescent, whereas chemiluminescent detection is more sensitive and produces signal at lower concentrations than colorimetric. Second, just as using a labeled 2°Ab boosts the signal compared with using a labeled 1°Ab, use of a labeled tertiary antibody can boost signal by providing additional amplification (Delaive et al., 2008).

Stripping and re-probing

The stripping and re-probing of WB membranes provides a time-efficient method for determining multiple protein targets within a single gel run (Sennepin et al., 2009). It allows a number of different analyses on a single membrane, thus saving time, sample and consumables, and maximizing efficiency. Furthermore, there may also be times where a membrane requires probing with a 1°Ab to confirm the data obtained from the initial analysis of the protein of interest (i.e., a different 1°Ab specific to another epitope on the same protein) (Kaufmann & Shaper, 1992).

Initially developed in the early 1980s, these methods involved incubation in either urea, β-MCE/BSA buffers (Erickson et al., 1982), or highly acidic glycine buffers (pH 2.2; Legocki & Verma, 1981) at elevated temperatures for long periods (up to an hour in some cases), in order to remove the 1°Ab and detection reagent (e.g., enhanced chemiluminescence [ECL]). Ultimately, multiple protein targets could be detected within a single sample/run, since the 1°Ab have been disassociated from their target proteins, allowing other 1°Ab to be added (Yeung & Stanley, 2009). Since these early experiments, stripping buffers have progressed rapidly and many commercial preparations exist which claim to be gentler (generally consisting of a mixture of SDS, glycine, and detergents; TBST) and work rapidly (<15 min) at room temperature. However, it is always recommended to check the efficiency of these commercial buffers, by re-exposing the membrane with ECL reagents or other detection methods post removal of the antibody.

Stripping and re-probing is commonly undertaken to investigate the levels of phosphorylated proteins before assessing the total expression of the same protein (Figueiredo & Nader, 2012), allowing the signaling capacity vs activation to be assessed (discussed below). In doing so, the generally weaker expressed phospho-protein is first detected, allowing accurate measurement, before the total is probed. Despite the obvious benefits of stripping and reprobing of membranes, these methods are not always foolproof and can have significant limitations. First, care must be taken as to which protein the membrane is re-probed for following stripping, as it is difficult to eliminate

detectable signal from highly abundant proteins. Additionally, if the stripping buffer has failed to completely remove all antibody and ECL from the membrane, and the two proteins of interest are of similar molecular weights [e.g., GAPDH (molecular weight: 35.8 kDa, but band commonly detected at 37 kDa) and ERK 1/2 (molecular weights: 42/44 kDa, respectively)], then the signal from the ineffectively stripped antibody may interfere with the subsequent detection and quantification of the second target protein. Further to this, stripping of the membrane cannot be performed indefinitely. As a rule of thumb, only three stripping incubations are recommended, due to loss of the antigen (Sennepin et al., 2009). This limits the number of targets that can be probed from the same membrane but is still worth doing considering the benefit of measuring two or more targets within the identical sample. There are, however, a number of recent developments which have aimed to enhance the capacity for multiple Ab detection from the same membrane. Sennepin et al. (2009) described a technique whereby instead of removing or stripping the HRP activity linked to the Ab from the membrane, this is instead irreversibly inhibited with hydrogen peroxide, to allow up to five different sequential incubations/detections to be performed on the same membrane (Sennepin et al., 2009). There are suggestions, however, that the exposure to such strong oxidizing conditions may alter certain epitopes, potentially affecting Ab recognition (Kaufmann, 2001). A similar procedure can also be performed using sodium azide, which has been proposed to avoid this epitope issue; however, the method requires lengthy incubations of upwards of 16 h compared to 15 min with hydrogen peroxide, and as sodium azide is known to inhibit HRP detection, it may interfere with subsequent detection (Kaufmann, 2001). As previously discussed, both colorimetric and chemiluminescent approaches may utilize HRP-conjugated 2°Ab, and both may be utilized separately in the same blot for different targets (Kar et al., 2012). Initially, the weaker target is blotted for using colorimetric 3,3′,5,5′-Tetramethylbenzidine detection before heating in a β-MCE containing buffer before re-probing for the additional target with ECL detection.

Care should be taken with these methods and conditions should always be optimized; in addition, it should also be demonstrated that stripping and re-probing does not adversely influence subsequent quantification. Thus, the undertaking of stripping and re-probing must be carefully considered as the probable loss of an antigen may in fact lead to erroneous measurements. This becomes more important when changes in protein expression are small or gradual (i.e., time-course experiments). However, the ability to measure both the phosphorylated and total expression of the same protein within the same blot is extremely desirable. Therefore, one must consider, when performing WB, whether it is appropriate to strip and re-probe a membrane, as despite the benefits (phospho vs total protein), the limitations may outweigh the benefit, and consequently the accurate detection and quantification of WB data may only be possible with repeated blots.

Analysis

Analysis of the bands of interest depends on the type of detection and the imaging system available, with many imaging systems requiring proprietary software for image acquisition and quantification. Each software package utilizes slightly different methods for quantification; however, generally peak height or area is used (Gassmann et al., 2009). During quantification, it is essential to ensure that the bands of interest are within the linear range of detection, as pixel saturation on imaging sensors may occur within highly abundant targets (Mollica et al., 2009); this is quite a common error and can easily be avoided. However, automatic detection of saturation is now a standard feature within most imaging packages. If oversaturation occurs, it may be possible for the sensitivity of the camera to be reduced (i.e., reduced pixel binning) increasing the image resolution (larger image, more pixels) and therefore requiring more signal to achieve oversaturation. Despite this, oversaturated "staining" of the membrane (i.e., as signified by visible yellowish bands on the membrane prior to exposure) during chemiluminescent detection by highly abundant proteins (e.g., GAPDH) may occur requiring the reduction of either sample loading or greater dilution of the 1°Ab.

Another important consideration for quantification is the choice of single band or whole lane boxed analysis, with the former suitable for individual protein analysis, and the latter better for the detection of global protein changes (i.e., ubiquitinated proteins or puromycin incorporation). Single-band analysis is undertaken simply through identifying sample lanes before determining the band of interest, while whole lane analysis will measure the intensity within a designated area (i.e., single lane) (Taylor & Posch, 2014). Whichever method is chosen, it must be consistent throughout the blot, with an equal area of analysis used per lane; otherwise different volumes (size of the analysis box region) will be analyzed producing potentially distorted data. Although WB conditions will have been optimized to produce the clearest bands possible, a visible background may still occur, requiring background subtraction during analysis (Gassmann et al., 2009). Principally, this is achieved through subtracting the calculated background density from peak values; however, the

different algorithms used to determine background intensity may not be truly representative. One common method is the rolling disk algorithm, which determines peak origins based on where a specified disk could "roll," typically the smaller the value (smaller disk), the greater amount of background becomes subtracted (Taylor & Posch, 2014). This approach is better suited to prominent individual bands since smaller, less intense bands may become lost. Another method is to manually designate a region of consistent background with no bands, assigning that density value for background correction. This method may be more suitable for whole blot analysis, with minimal fluctuation in background levels. Ultimately, the choice of analysis must be representative of the visualized blot and within the range of linear detection of the imaging method employed.

Normalization of target protein abundance

To account for possible errors in sample preparation and loading, normalization of samples to remove inter sample/gel variation is paramount. It is typical for specific "housekeeping" proteins (HKP) such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin to be probed for, acting as internal loading controls assuming their expression remains stable under the experimental conditions used (Welinder & Ekblad, 2011). The HKP chosen should be one that is known to stay constant between control and experimental samples and demonstrated to be unaffected by the treatment or intervention undertaken. Errors such as loading more sample within one well will increase the target signal, likely skewing data interpretation. As such, target measurements may be normalized to HKP values, removing loading bias. However, the accuracy and effectiveness of these HKPs are dependent on multiple factors such as oversaturation of the protein, high background, and lack of linearity (McDonough et al., 2014) and can easily suffer from technical errors within the WB process (Eaton et al., 2013). One study has demonstrated the linear range of up to 5 μg of loaded protein when probing for GAPDH, showing no increase in band density at higher concentrations (Welinder & Ekblad, 2011). Other common loading controls (e.g., pan-actin and β-tubulin) have also displayed poor linear ranges in the same manner (Li & Shen, 2013). This lack of linearity may be resolved by loading less protein but may reduce the sensitivity to detect lower abundant proteins. Expression of HKPs such as β-actin has also been shown to be extremely variable between tissue types (i.e., muscle, heart, fat) (Eaton et al., 2013). Alarmingly, however, expression of β-actin has been revealed to not be homogeneous within a single tissue

sample, being shown to differ between proximal and distal regions of a single mouse sciatic nerve (Eaton et al., 2013). Such differences in expression may become more problematic within skeletal muscle as it is a large complex tissue, with different regions potentially responding differently to stimulation (Seynnes et al., 2007), therein WB results only provide an average of expression changes within a single sample. The use of a second HKP may help; however, some of the same issues may exist and it will need to be demonstrated that the choice of HKP does not affected the interpretation (Fig. 2). As a result of the potential changes in HKP expression in response to the experiment and the limited linear range of some, the use of HKPs for normalization may mask or confound potentially relevant changes in protein expression.

A viable alternative to blotting for HKPs is to assess the total amount of protein either within stained or stain-free gels or on stained membranes (Welinder & Ekblad, 2011; Eaton et al., 2013). Assessing total protein offers distinct advantages over HKPs as it is unbiased with respect to changes in the expression of a single (or multiple) HKP, and if utilizing stained membranes, also allows evaluation of the blotting process and transfer quality (Taylor et al., 2013). Coomassie staining a gel and fluorescence detection has shown the existence of a wide linear range (1–40 μg), negating previously discussed HKP linearity issues. Membranes may be stained to visualize total protein by several methods (i.e., Ponceau S, colloidal silver, India ink); however, Coomassie staining is a common, simple approach that has been demonstrated to be an unbiased method of total protein assessment (when analyzing total lane volume) with a high linear range of detection (2.5–25 μg) (Welinder & Ekblad, 2011). If one of these approaches is chosen, the quantification of a single random band that is consistent across each lane may be used for normalization. In our experience, Coomassie staining a membrane is an effective
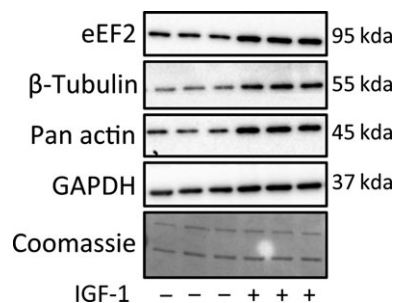

Fig. 2. Representative blots for multiple housekeeping proteins and the Coomassie-stained membrane of IGF-1-treated C2C12 cells, demonstrating potential variability in housekeeping proteins in response to a treatment.

method of providing reliable quantification of total protein that removes potential problems associated with individual HKP expression. Staining the membrane or gel allows appropriate quantification normalization for whole lane analysis providing more robust normalization approach for both ubiquitinated proteins and puromycin incorporation, as discussed earlier.

The final aspect of analysis is the requirement to calculate the changes in protein expression, resulting from a treatment/intervention. Depending on the experimental design, multiple blots may be required to analyze all samples, and thus, subtle changes within the process may influence the final data as background or band density may be variable across multiple blots. In this instance, a single quality control sample (typically pooled from multiple controls) is loaded on each gel, providing a control sample across all gels, allowing gel-to-gel comparisons to be made. Dividing band values (i.e., band density) by the quality control sample (as a correction factor) normalizes differences in loading, separation, transfer, and detection that may have occurred. This should be undertaken for the values of the initial protein(s) of interest (e.g., Pan-AKT, p-AKT Ser 473, or puromycin incorporation), along with the normalization values (i.e., HKP or total protein). Another approach typically used for human studies is to use an initial basal sample for each individual to assess changes in subsequent samples, permitting the calculation of a fold change from the initial basal levels. Whichever approach is chosen, it must be applied consistently throughout analysis and may depend on the total number of samples and comparisons needed (i.e., control vs treatment or control vs treatment 1 vs treatment 2).

As previously mentioned, measurement of both the total expression and phosphorylation status of a protein (either by multiplexing fluorescent 2°Ab or stripping and re-probing) is important. Determining changes to a protein's total expression in response to a treatment or intervention indicates its capacity to signal, since a greater abundance or protein will allow a greater potential for signaling to occur. Measurements of a protein's phosphorylation status (i.e., signaling activation) may change through modification of the individual proteins phosphorylation level (Tremblay et al., 2007) or by alterations within the total amount of protein available (Yung et al., 2011). Consequently, the normalization of a phospho-protein to its total expression allows the ratio of phosphorylated proteins to be assessed (i.e., the relative proportion of phosphorylated vs non) (Wilkinson et al., 2008). For example, within skeletal muscle, the total expression of various anabolic signaling intermediates (e.g., AKT, P70 S6K1) remained unchanged, while phosphorylation of these proteins increased (Brook et al., 2015). As such, the ratio of phosphorylated proteins to total expression increased, demonstrating the increased proportion of phospho-proteins. Importantly, however, if the treatment undertaken increases both total and phosphorylation levels, this ratio may remain unchanged, masking any potential mechanisms, and thus it may be extremely important for both measures, i.e., total and phosphorylation status, to be made simultaneously.

Presentation of representative blots

One essential aspect and ethical concern in the reporting of scientific findings is the accurate and representative presentation of WB data and example images (for a more in-depth review see Rossner & Yamada, 2004). Generally, WB data are presented as both a graphical and representative image to demonstrate the effect of the intervention and the quality of blot. Herein lie ethical considerations in presenting images that are required to present images that accurately represent the quantified data (Rossner & Yamada, 2004). For example, the splicing of images from multiple blots to form one continual image may obscure the magnitude of change between samples. If possible replicates from all experimental groups should be run within the same blot to provide a representative image; however, if this is not
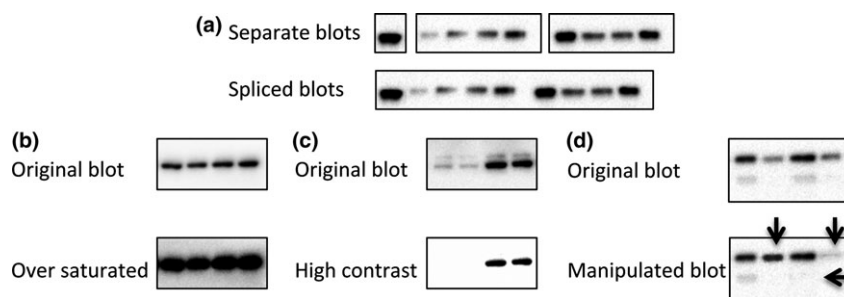

Fig. 3. Examples of image manipulation techniques upon representative blots. (a) Multiple blots spliced together to give the impression of a single blot. (b) An oversaturated blot with no clear distinction between bands of interest. (c) Contrast adjustment masking additional bands. (d) Image manipulation to intensify, reduce, or remove bands.

possible, clear distinction between blots should be made (Fig. 3). Additionally, sample replicates should be included within the images, demonstrating equivalent changes in response to the intervention. The inclusion of a representative loading control (i.e., Coomassie) is essential to demonstrate that changes in samples are due to the intervention, rather than protein loads. These are required to be from the same blots as the other representative images and should not be repeated unless the same gel has been stripped and reprobed for multiple targets or different molecular-weight targets were measured. As previously discussed, one method of validation of target specificity is the knowledge of a correct molecular weight. Despite this, it is common to crop representative blots to reduce their overall size. The addition of an indicator of the precise molecular weight must be included even if image cropping occurs to allow identification. For example, when blotting for phosphorylated 4E-BP1, three isoforms typically form distinctive bands (i.e., γ, β, α) at differing molecular weights (Elia et al., 2008). In this instance, each isoform should be indicated with the appropriate molecular weight. Accordingly indicators of molecular-weight markers should be included to allow the independent determination of a targets size and specificity.

Representative images should clearly show individual bands that are not over loaded/saturated. As with any representative image for scientific publication, inappropriate manipulation may be regarded as misconduct. Thus, manipulation of an image to change the intensity of a band(s) or even to remove it altogether is highly unethical (Fig. 3). If any alteration of an image is made, it should affect the entirety of the image (rather than a specific section) and be clearly stated in the figure legend. Finally, the inclusion of detailed information of the source of both the primary and secondary antibodies is essential for reproduction in other laboratories along with possible future discussion of antibody specificity.

Alternative and emerging methodologies to WB

Although this review focuses on the technical aspects and choices to be made to undertake robust and accurate WB measures, it is often important to utilize additional complementary techniques to supplement and support the data that WB generates. One common approach is the use of an ELISA to quantify the abundance of a target protein (total or phospho) (Timen et al., 1976) although this method can be expensive, requiring access to a plate reader. Commonly, ELISAs use sample lysates containing non-denatured native proteins (Dhingra et al., 2011); however, this may not always be the case. ELISAs offer distinct advantages such as being in

96-well plate format, increased sensitivity, relative ease of absolute protein concentration (using supplied standards), and reduced time consumption (2–4 h), proteins of varying molecular weights cannot be distinguished from one another, and this is a crucial consideration that must be made as the 1°Ab used may bind to multiple proteins producing a falsely high signal. Additionally, ELISAs are not capable of re-probing once completed, instead require multiple plates to be run, thus potentially requiring more sample than multiple WBs. Another emerging technology is the use of protein arrays to detect the presence of multiple target proteins within a single sample (Huang et al., 2001), to explore potential protein interactions with other proteins, and even DNA and RNA, and to measure enzyme activity. Utilizing the same principle as WB and ELISAs, arrays typically contain individual spots of labeled 1°Ab immobilized to the array for multiple targets. Although WB can provide important information on modulation of signaling networks, potential targets (or pathways) must first be identified, and developments within array kits allow the probing of dozens of targets simultaneously within a single sample, providing lots of data with relative ease. Interesting observations may then be further investigated using traditional WB techniques. Although the use of ELISAs is now commonplace, the use of mass spectrometry (i.e., LC-MS/MS) for the analysis and extremely accurate quantification of proteins is becoming more widespread (Ryder et al., 2015). Mass spectrometry offers distinct advantages over both ELISAs and traditional WB techniques as the distinction between multiple protein isoforms is possible with a high degree of sensitivity and over a wide dynamic range (0.5–500 ng/μl) (Hu et al., 2005). However, such analysis requires highly specialized, expensive equipment and technical expertise not commonplace within most labs. Mass spectrometry can be used in conjunction with WB to undertake highly specialized measures, e.g., to confirm antibody specificity, to determine novel protein–protein interactions after IP, and to uncover PT modifications (Mann & Jensen, 2003).

Conclusions

WB has emerged as an essential tool within physiological research; nevertheless with poor understanding and implementation, any subsequent analysis can produce misleading and confusing interpretation (i.e., Ab specificity and validation). Before a sample is loaded into a gel, careful consideration must be given to often overlooked aspects such as the appropriate buffer for homogenization and extraction of the intended target protein for denaturation. Gel composition should effectively separate proteins by

size, with changes to concentration giving resolution to the intended target by varying migration speed. Subsequent transfer onto an immobilizing membrane will allow the probing for one or more targets with 1°Ab and 2°Ab, with emphasis upon Ab specificity and the ability to assess PT modifications. Validation of Ab should always be undertaken, using both positive and negative controls to try to ensure specificity. Within each study design and group comparisons, QC samples should be used allowing the comparison of multiple gels. The method of detection will be ultimately be determined by the equipment available. However, fluorescent antibodies have a greater dynamic range and may be multiplexed for additional targets if desired. As sample quantity may be scarce, the ability to strip and re-probe membranes for additional targets is desirable; however, potential issues with regard to quantification and potential signal reduction should be considered carefully and where possible mitigated. Finally, the quantification and analysis of band intensity should be evaluated consistently throughout with both single and multiple blots; as doing so can produce reliable and accurate data.

Perspectives

Western blotting techniques are now considered routine inclusions within the field of physiology and are invaluable in providing mechanistic insight into many regulatory processes. The present review aimed to give a comprehensive insight into the multiple aspects within the WB process, providing the reader with enhanced expertise to critically evaluate and troubleshoot all features to produce reliable and reproducible blots.

Key words: Westen blot, physiology, SDS PAGE, skeletal muscle.

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