GOALS AND PRACTICALITIES OF IMMUNOBLOTTING AND IMMUNOHISTOCHEMISTRY: A GUIDE FOR SUBMISSION TO THE BRITISH JOURNAL OF PHARMACOLOGY

Steve P.H. Alexander, Richard E. Roberts, Brad R.S. Broughton, Christopher G. Sobey, Christopher H. George, S. Clare Stanford, Giuseppe Cirino, James R. Docherty, Mark A. Giembycz, Daniel Hoyer, Paul A. Insel, Angelo A. Izzo, Yong Ji, David J. MacEwan, Jonathan Mangum, Sue Wonnacott & Amrita Ahluwalia

Correspondence: A. Ahluwalia, British Journal of Pharmacology, The Schild Plot, 16 Angel Gate, City Road, London EC1V 2PT, UK. Email: info@bps.ac.uk

Reproducibility is a current concern for everyone involved in the conduct and publication of biomedical research. Recent attempts testing reproducibility, particularly of note the reproducibility project in cancer biology published in elife (https://elifesciences.org/collections/9b1e83d1/reproducibility-project-cancer-biology), have exposed major difficulties in repeating published pre-clinical experimental work. It is thought that some of these difficulties relate to uncertainty about the provenance of tools, lack of clarity in methodology and use of inappropriate approaches for analysis; the latter particularly related to untoward manipulation of images. In the past, some of these so-called untoward practices were considered the 'norm', however, today the landscape is different. The expectations, not only of the readers of the published scientific word, but of the publishers and funders of research, has changed. This collective group now expects that any published data should be reproducible; but for this to be possible, experimental detail, confirmation of selectivity and quality of reagents/tools, analytical and statistical methods used need to be described adequately. Two powerful methodologies often used to support researchers' findings allow the detection of changes in protein expression, i.e. immunoblotting (widely known as western blotting) and immunohistochemistry. Undeniably, as a result of unintentional mistakes (often related to lack of antibody specificity (Baker 2015)), but, in some cases, deliberate alterations and questionable interpretations of results, the use of these two methods has led to many high profile retractions. Indeed, such images have driven the retractions that have occurred in BJP over the last two years.

Today immunoblotting and immunohistochemistry serve as primary methodologies for the detection and quantification of molecular signalling pathways and identification of therapeutic targets. This necessitates clear guidance for the application of these techniques, the need for controls (both positive and negative) and the most appropriate methods for quantification. Indeed this need has spawned a number of initiatives to support researchers in assessing the validity of antibody resources including antibodipedia (Bjorling and Uhlen 2008) and the resources available within 'The Human Protein Atlas' (Thul et al. 2017). The aim of this article is to outline the rationale for, and the expectations of, the BJP with respect to work published in the Journal that includes immunoblotting or immunohistochemical data. In creating these guidelines, our aim is to reduce potential misinterpretations and to maximise the communication and transparency of essential information, particularly with respect to the methodologies employed.

We have generated the guidelines below for the benefit of authors, editors and reviewers. Whilst we recognise other recently published guidelines (Uhlen et al. 2016), and indeed we have incorporated some of the advice provided in such works, we focus on the evidence required for publication in BJP. These guidelines join a series published in BJP regarding the reporting of animal experiments through adoption of the ARRIVE guidelines (McGrath and Lilley 2015; McGrath, McLachlan, and Zeller 2015), experimental design and analysis (Curtis et al. 2015) as well as data sharing and presentation (George et al. 2017) in preclinical pharmacology. We would be delighted if other journals were to also use these guidelines.

METHODOLOGICAL CONSIDERATIONS ENABLING REPRODUCIBILITY OF IMMUNOBLOTTING AND IMMUNOHISTOCHEMICAL DATA

To enable a reader to make an objective judgement about the importance of any immunoblotting or immunohistochemical data, a number of key (necessary and sufficient) pieces of information should be provided in any manuscript published in BJP so as to enable independent reproduction of the findings:

- The antibody species (mouse monoclonal, goat polyclonal, *etc*) and isotype (IgG, IgY, *etc*).
- The source (commercial company or donor) and catalogue and lot numbers, where appropriate.
- The epitope against which the antibody has been raised.
- A description of the secondary antibody (species, isotype and 'tag').
- Final dilutions of each antibody and the buffer(s) in which they have been prepared.
- Whether solutions were re-used, how they were stored and how many cycles of re-use. For example, "dilutions of secondary antibody were maintained at 4 °C and re-used up to five times".
- The source and grade of blotting membrane.
- The blocking methodology, including the concentration and source/grade of albumin/milk powder/fish gelatin/etc.

If an antibody has been characterised previously, a citation must be included. This characterisation should fulfil the requirements detailed in the recent guidelines generated by 'The International Working Group for Antibody Validation' and should take the form of at least, but preferably more than, one of the 'five conceptual pillars of antibody validation' described in the guidelines (Uhlen et al. 2016). We also ask authors to check currently available databases for known issues with selectivity. Currently, we highly recommend the NIH-established Research Resource Identification Protocol (RRID) at https://scicrunch.org/resources/Cell%20Lines/search, which provides a unique identifier for the antibody that enables reproducibility studies through clear indication of provenance. Although encouraged, the inclusion of an RRID is not currently mandatory for BJP; we envisage that a similar level of individualisation will become essential for publication in the near future.

Critical importance of controls

To support the validity of conclusions emanating from immunoblotting or immunohistochemical observations (as with the majority of biological assays), it is important to conduct both positive and negative controls--although for some proteins, certain positive and negative controls are unknown or such materials are not available, thereby precluding such assessments. However, there are a number of other 'method' controls that are always possible and which should be included in assay protocols. We recognise that there may be no perfect control that provides confidence beyond doubt (Torlakovic et al. 2015).

The key issue for antibody-based methodologies is the selectivity of the antibody. If the antibody has not been previously characterised in the species and/or tissue/cell in use, appropriate controls must be included in any submitted manuscript. At BJP, we accept that the priority for each of the controls listed will vary depending upon previous information (for which citable evidence must be available within the public domain). Below we list factors that we have identified as critical for establishing confidence in specificity:

- Use of material from genetic controls. Thus, one should compare wild-type and gene-disrupted mice from the same strain/genetic background or transfected versus empty vector-transfected cells. This enables comparisons to check correct/similar molecular weights or cellular localisations
- Controls in the absence of the primary antibody
- Where possible, controls utilising 'pre-immune' serum
- If available, a blocking peptide should be applied, i.e. to confirm the epitope against which the antibody was raised
- A dilution curve of the primary antibody should be conducted and described

Method-specific considerations for immunoblotting

In addition to essential information regarding antibody protocols and blocking, specific methodological issues relevant for immunoblotting should be considered. Those most pertinent for BJP are as follows:

- *Sample preparation*: extraction-associated protein modification should be minimised (for example, by the inclusion of inhibitors of proteases and phosphatases). Samples should be handled in an identical manner, for example, in the isolation, extraction and storage (duration and temperature of storage should be indicated as well as any freeze-thaw cycles).
- *Molecular weight markers*: these are essential control elements required for every gel/blot and will form part of the checklist (see below) for passing review in the BJP.
- *Loading controls*: these are a critical requirement for quantitation of immunoblotting, in particular if authors are attempting to demonstrate the absence of a protein, or selective alteration in protein expression. This loading control is crucial to demonstrate that changes in expression of the protein of interest are not due to a difference in the total protein within that particular sample, or a difference in loading on the gel. The choice of the protein to use as the loading control is also important. β-actin and GAPDH are commonly used, since it is thought that expression of these proteins is less likely to change, but expression of these proteins can change under certain conditions. Authors should thus ensure that the most appropriate control has been selected and should provide evidence for consideration of this issue.
- *Band analysis:* multiple bands may be present due to biological or artefactual modification of proteins. Unless there is a good reason to exclude individual immuno-positive bands (such a reason should be clearly stated), all bands should be included in the analysis.
- *Signal linearity*: for quantitation, numerical evaluation of band intensity (usually assessed as optical density or fluorescence units) for the protein/s of interest and loading controls should correlate linearly (over the range employed) with the protein mass applied to the gel. This is best achieved with a standard curve run over a wide dynamic range at the start of experiments with a new antibody or detection methodology. This is of particular importance where signal saturation can occur, for example, with enhanced chemiluminescence (ECL)-type visualisation.

Method-specific considerations for immunohistochemistry/immunofluorescence

As with immunoblotting, when a manuscript includes immunohistochemical/fluorescence data, BJP requires evidence of consideration of a number of method-specific technical issues:

- *Autofluorescence:* Cells and tissue contain many proteins and compounds that are intrinsically fluorescent when excited by light of particular wavelengths. Within the cell, the primary source of autofluorescence is from the mitochondria and lysosomes (Monici 2005). In addition, collagen and elastin contain several endogenous fluorophores and contribute substantially to autofluorescence. A third source of autofluorescence may arise by the formation of Schiff bases if the fixation process with aldehydes is extended (Hoffmann et al. 1993). It should be noted however, that autofluorescence typically appears dim and relatively uniform when compared to antibody-directed immunofluorescence. To image or analyse the amount of immunoreactivity accurately within a sample, it is important to eliminate the contribution from autofluorescence. The simplest way to do this when imaging is to reduce exposure time and fluorescence gain in 'unlabelled' samples until it disappears. Alternatively, commercially available products, such as FocusClear and TrueBlack, can limit the amount of autofluorescence. However, if it is unavoidable, autofluorescence should be measured and subtracted (Rietdorf and Stelzer 2006).
- *Bleed-through (or cross-talk):* The accuracy of immunohistochemical co-localisation relies on one's ability to distinguish between the various fluorophores. When imaging a specimen with two (or more) fluorescent labels it is important to prevent or correct for fluorescent bleed-through. Detection of fluorophore's signal can appear through the filter set of another fluorophore; this is problematic when a sample is incubated with multiple fluorophores with overlapping excitation and emission profiles (Rietdorf and Stelzer 2006; Waters 2009; Bolte and Cordelieres 2006; Ploem 1999). Consequently, the immunoreactivity appears identical for each of the fluorophores. To limit

the likelihood of crosstalk, investigators must carefully select fluorophores whose emission spectra are further apart, with little or no overlap (*e.g.,* Alexa 488 and Alexa 594 or 633).

- *Exposure time:* Choosing an optimal exposure time, which is maintained for every sample, is essential for imaging. The aim is to take an image that mirrors the level of fluorescence observed under the microscope. Increasing the exposure time to enhance the immunofluorescence above that observed under the microscope so increases the likelihood of imaging autofluorescence and/or bleed through, as well as the risk of oversaturating the image and fading of the fluorophore. If pixel saturation occurs, these images cannot be meaningfully quantified. Moreover, for the quantification of immunofluorescence, to ensure consistency, it is critical that image settings are identical for all samples. Thus, the ideal is to perform a single immunohistochemistry experiment that incorporates all the samples to be compared so as to ensure they are all exposed to the same environmental/protocol conditions.

IMAGE PRESENTATION CONSIDERATIONS FACILITATING INTERPRETATION

Image presentation

In this digital age a growing concern amongst the scientific community is that images for publication are not being accurately presented, i.e., the problem of unintentional and/or inappropriate manipulation of images (Cromey 2010). For example, whilst it is usually acceptable to crop an image to simplify the information, choosing to crop out oversaturated regions and/or areas/bands displaying non-specific immunoreactivity is not acceptable. BJP requires submission of full immunoblot scans and immunohistochemical/fluorescent images, from which figures have been generated. These scans/images should be included as an additional file for the review process and will be used by the Editors and reviewers when assessing the manuscript, but need not be published. In instances where uncertainty occurs regarding image compilation and assembly during the review process, the BJP Editorial Office makes use of freely available Office of Research Integrity Forensic tools (https://ori.hhs.gov/forensic-tools), developed by the US Department of Health and Human Services.

We require authors to present some general attributes of any scanned image presented in any figure as part of the submitted material:

- Any scan of an immunoblot shown in a figure should be as complete as possible. If the blot has been cut, this must be clearly stated in the methods together with a justification. An acceptable reason for so doing might include probing different regions of the same blot with multiple antibodies (the full blots should be submitted for review purposes).
- For immunoblotting, the figure should indicate the position of the molecular weight markers; at a minimum, the bracketing of molecular weight marker sizes (and the markers themselves, where practicable) on either side of the band of interest should be shown.
- For immunohistochemistry/fluorescence, ideally a lower magnification image should be presented with higher magnification images (usually as an inset to represent the target of interest). If this is not possible, then the higher magnification image should be provided as an additional file for review purposes. Such images help verify that the immunoreactivity is specific for the target of interest and/or that the specimen is not overexposed so as to display sufficient immunoreactivity.

Conforming to these stipulations will ensure that reviewers and readers can confirm that the band identified is at the correct molecular size (or allow some interpretation of post-translational modifications, for example) and to determine the selectivity of the antibody used.

Image analysis

Immunoblots:

- What is the best housekeeping protein to use? For changes in post-translational modification, the loading control should be the total protein (for ERK phosphorylation studies, total ERK is commonly probed in parallel).

- Normalisation to the loading control: If authors express the density of the bands obtained with anti-phosphoprotein antibodies relative to bands obtained with anti-total protein antibodies, such comparisons must assess samples on the same blot. One issue with normalisation to loading controls is that the samples may have to be run on separate gels, particularly if using ECL-type visualisation. For fluorescence-based systems, such as the Odyssey system (Licor), it is possible to assess the loading control on the same gel as this system enables the use of two different secondary antibodies and detection at two separate wavelengths. However, this depends upon having antibodies from two different species. Bands obtained from proteins run on two different gels cannot be subject to normalisation. In this case, authors should present the densitometric readings from the loading controls separately from the readings from the protein of interest.
- It is essential that any comparisons between bands are done within the same blots. Quantitative comparison of proteins on different blots is not appropriate, as differences in antibody concentrations, transfer efficiency, incubation time and conditions could alter the density of the bands. Control samples should be run on the same gel and comparisons made between samples run on the same gels.
- If lanes are missing or a blot contains data not relevant to the manuscript, best practice would be to re-run the gel to generate a publication-acceptable blot, although this may not always be possible. For publication purposes, these extra bands can be cut out, provided that it is made clear in the legend that this has been done and that the bands presented in the figure are from the same blot. Bands from different blots should NEVER be presented together.

Immunohistochemical images:

Analysis of histochemical images can be very subjective. Thus, it is imperative that efforts be made to avoid potential bias. A number of simple methods can limit bias. BJP requires that such approaches be used and specified within manuscripts:

- Blind analysis. We realize that in some cases blinding of *in vitro* or *in vivo* experiments is difficult, and may require multiple personnel. However, the blinding of analysis is often logistically a much simpler process. We expect that blinding during image analysis stages is routinely conducted, perhaps by anonymisation of images or through use of automated computer-based processes.
- Where automated methods have been used to limit bias, full details of the parameters set for this automation should be provided. Automated segmentation methods are available using freely available software such as ImageJ (http://imagej.net/Principles) or MyoScan (Pertl et al. 2013).
- If, for some reason, these approaches cannot be undertaken, then a justification must be included in the manuscript. Often, focussed regions of antigen expression are present within the section/culture/cell. Thus, quantification of expression needs to account for the possibility that an intervention might alter the number of regions of expression within a section and/or the amount of expression within a specific region of localization.

BJP GUIDELINES FOR IMMUNOBLOTTING/IMMUNOHISTICHEMISTRY- A CHECKLIST FOR AUTHORS, REVIEWERS AND EDITORS

We expect that future submissions to the BJP should include, either in the Methods or Results section, as appropriate:

1. For any antibody (including secondary antibodies) used, the Methods section should include the following:
    a. The commercial (or other) source
    b. The species in which the antibody was raised, catalogue and batch/lot numbers
    c. The epitope against which it was raised
    d. The isotype (IgG, IgM, IgY, *etc*) and clone numbers (if applicable)
    e. When available, RRID
    f. The diluting buffer, the final antibody dilution and number of times solutions have been re-used
    g. The type, grade and source of the blotting membranes
    h. The concentration and source/grade of blocking protein/s
2. Positive and negative controls should be used, as much as possible
3. Full uncropped images should be made available to reviewers and editors.
4. Separate immunoblots should NEVER be merged in figures.
5. Normalisation to the loading control should be done only if the bands for the target protein and the loading control are obtained from the same blot.
6. Quantitation of band density can only be conducted on analysis within the linear range
7. Statistical comparisons should only be carried out between bands on the same blot.
8. Full details of blinding for analysis of images should be provided.

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