



Validation of Immunohistochemical Outcomes on Frozen Sections Critical for Reliability and Reproducibility

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Immunofluorescent staining has been a powerful tool in visualizing cell types, proteins and biological polymers for over 80 years (Coons, 1960). Since its inception there has been tens of thousands of important scientific questions answered with its aid. In many cases immunofluorescence can even be performed years after the specimen has been frozen. This allows for follow-up studies to help answer additional questions. But as the specimen ages the molecular targets degrade. Thus, sometimes immunohistochemistry is still possible but other times it is not. The reliability of these stains must be validated for reliability. I report one instance in which validation through multiple methods prevented improper conclusions.

My purpose in the Drummond lab for the last 10 weeks has been to characterize the immune response in skeletal muscle to 5 days of bed rest with administration of neuromuscular electrical stimulation and protein supplement (NMES+PRO). This analysis utilized immunofluorescent staining on sections taken from human *vastus lateralis*. These sections had been cut and mounted on microscopes between 1 and 3 years ago. The stain consisted of primary antibodies for the macrophage markers CD11b and CD206 and secondaries with emission wavelengths of 488 nm (green) and 568 nm (red), respectively. Due to a weak signal, the secondary antibody used to stain CD11b was biotinylated which allowed for amplification with horseradish peroxidase and an AF 488 conjugated to tyramide. The CD206 marker was vibrant, with the lookup table (LUT) in Nikon's imaging software, NIS-Elements, maxing out between 5000 relative fluorescence units (RFU) and 10000 RFU. The CD11b marker, despite efforts to amplify the signal, was very faint with the LUT reaching only 4000 RFU. This effect was observed in each section. It appeared that the CD11b marker had degraded despite efforts to preserve it.

To investigate possible reasons that one marker would degrade and the other would not we considered biochemical factors. This phenomenon has not been explained in this exact situation but we speculated that structural differences between the markers are responsible for the differential preservation between them. CD206 is a transmembrane protein with a glycosylated cap. Sola *et al.* showed that glycosylation is capable of protecting proteins against the harsh environment they are often found in. This, and the transmembrane portion on the other end, may be important in the preservation of the receptor. CD11b on the other hand is not glycosylated and is part of a much larger structure. There are also no intracellular domains to provide any anchoring. The lack of stabilizing structures on CD11b implies a shorter life span when compared to CD206 and would explain the vibrant red and the dim green reflective of the respective preservation and attenuation of those antigens.

Our attempt at staining was performed on 10 μm thick sections that had been stored in a -20 C freezer for several years. When the biopsy specimens, from which the old slides were cut, were removed from the -80 C freezer, recut and mounted on new slides, the green CD11b stain

had similar intensity to the CD206 (Figure 1). This confirmed our suspicion that the older sections were unreliable when staining for that particular marker.

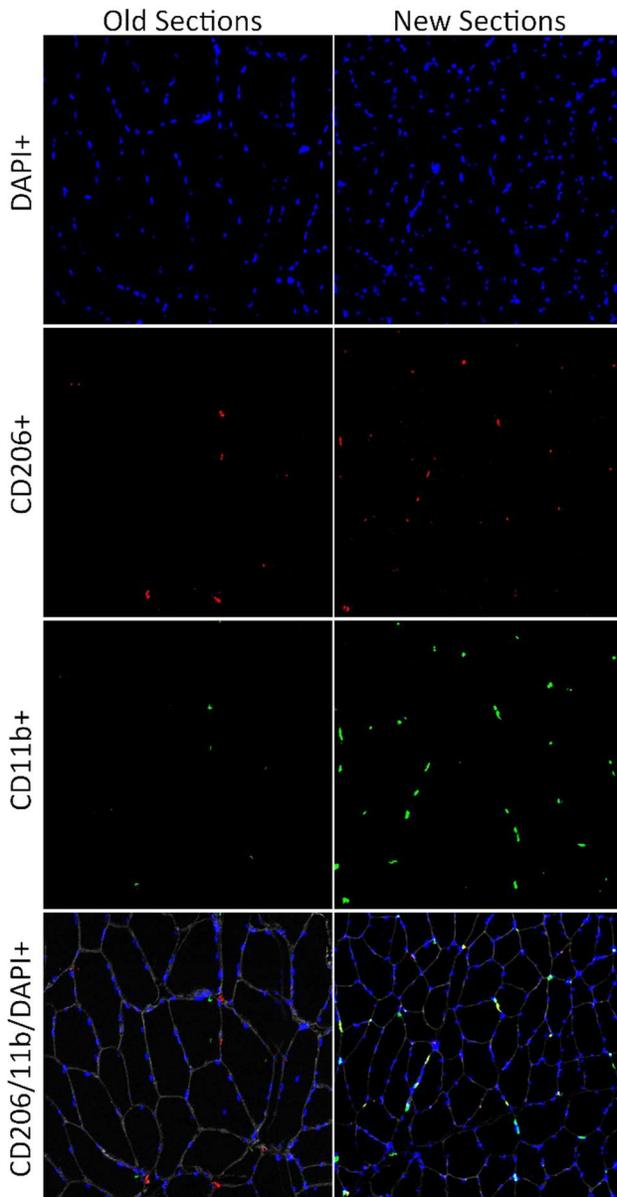


Figure 1: Representative images of the stains used when marking macrophages. DAPI+ (blue) is a nuclear stain. CD11b (green) and CD206 (red) are macrophage markers. A laminin stain (gray) marks the borders between muscle fibers.

Previous literature existed detailing the protocol used which also contained representative images (Kosmac *et al*, 2018, Ballotta *et al.*, 2014). Were it not for these images there would have been a much higher chance that the reliability of the stain on old sections would not have been questioned. Therefore, it is absolutely expedient for the reputation of the scientists involved and the integrity of the field to confirm the results of the assay being used. If a new assay is being developed the scientists should have an idea of what to expect as far as quantity, magnitude of signal and morphology. This can be done through mathematical modeling, computational simulations, controls or simple logic depending on the situation. Our controls are found in a paper by Reidy *et al.* published in 2019.

When an assay does not come out the way expected there are a number of reasons why. One reason is that the assay is in fact valid. It is up to the scientist and their expertise to decide if this is true. The decision should be informed by biochemical considerations (like glycosylation, membrane binding etc.), related peer-reviewed literature and after running any control procedures required to make it.

Reliability is the foundation of reproducibility, and many conclusions made by original researchers are invalidated due to the inability of others, or themselves, to reproduce their data. Scientists that persistently produce irreproducible data quickly lose their standing in science and inevitably face problems in funding their work. Therefore, though it may take extra time, all techniques must be validated before publication.

We, as scientists, are in the midst of a “reproducibility crisis.” In a news article published by *Nature* in 2016 it was reported that the majority of scientists have been unable to reproduce the data of other researchers. And just over half of them couldn’t reproduce their own data (Baker, 2016). The “publish or perish” culture that has been pushed into the field of science by funding organizations such as the institute at which the scientist is employed, the National

Institutes of Health (NIH), the National Science Foundation (NSF) and other foundations encourages failure to validate; and this must be addressed. But before this can happen, mentors must train their students to be aware of any potential issues with reliability and show them how to validate their findings. Doing so will help mitigate the “reproducibility crisis” and encourage higher quality publications in the future.

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