Quantitation in Immunohistochemistry: a Response.

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I have read with great interest the considered article by Dr. Barry Rittman (*Microscopy Today* #98-8, October, pp 8-9). In describing the difficulties of quantitation in immunohistochemistry, Dr. Rittman has gone to great lengths to give an authoritatively balanced view. It is, therefore, with some trepidation that I raise a few issues which may remain unresolved.

Dr. Rittman provides a list of criteria to be considered before applying quantitative analysis to this method. I would place the question of relating color developed to the amount of substrate present higher than number 7 on the list. To the best of my knowledge, there is little evidence that immunocytochemistry is stoichiometric, *i.e.*, that there is a relationship between color developed and amount of substrate present. Indeed many consider that it is not, which represents a serious drawback.

Producing sections of constant thickness is another pitfall to which Dr. Rittman draws attention. Not only is section thickness tedious to establish, but Helander (1982) has elegantly shown that the minuscule arc of vibration described by the knife edge as it "cleaves" the section is sufficient to glide over the surface of some nuclei, undermine others and successfully cut through the remainder. If those results are valid, then there are obvious difficulties when measuring the "concentration" of nuclear antigens. If there is an even distribution of this artifact, then presumably it may be ignored.

I also question the degree to which it is useful to quantitate the reaction, as determining the "optimal" concentration of the antibody used is based on a subjective assessment as the operator tries to balance unwanted background staining and maximum intensity of "specific" staining.

If quantitation is to determine the number of cells stained, rather that the intensity of the reaction, then the stoichiometric argument raised above becomes far less relevant. The problem will become one of degree and determining the level of accuracy that is important. Does it matter, for example, whether the difference is between a) 30% positive cells with 70% negative and b) 45% positive with 55% negative? More importantly, is it of clinical significance if the result is 43% positive or, say, 37% positive? I doubt it, although the degree of precision required for clinically significant reasons has yet to be determined. It may be that it is sufficient to "eye-ball" the section for an approximation.

Finally, achieving complete standardization of immunoreactions seems to me to be extremely difficult. It may be possible, as Dr. Rittman suggests, to standardize the fixation of gelatin blocks, but surgical specimens are an altogether different proposition. Personal experience in comparing the reactivity of tonsil using the same size blocks and identical times of fixation, does not produce identical fixation. The individuality of each block, except just possibly with liver, is marked. Differences arise because penetration and fixation using formalin (for example) do not proceed apace, penetration being much quicker (Medawar 1941). Identical times of exposure to formalin does not necessarily equate with identical fixation.

We have drawn attention to some of these idiosyncrasies in a recent paper on oral squamous cell carcinomas using as examples the antibodies p53, PCNA and Ki67. The results are bewildering!

Hopefully, this letter will contribute to a wider debate on the value of quantitation in immunohistochemistry, not merely emphasize the difficulties which we have found.

References:

Helander, K.G. 1983. Thickness variations within individual paraffin and glycol methacrylate sections. J. Microscopy. 132:223-227.

Medawar, P.B. 1941. The rate of penetration of fixatives. J. Royal Microsc. Soc. 61:46-57.

Allison, R.T. and T. Best. 1998. p53, PCNA, Ki-61 expression in oral squamous cell carcinomas: the vagaries of fixation and microwave enhancement of immunocytochemistry. J. Oral Pathol. Med. 27:434-440.

A Response to the Response:

Barry R. J. Rittman, The University of Texas

The purpose of my previous article was to identify many of the potential sources of error to be considered when evaluating quantitative data from immunohistochemistry (IHC) and histochemical reactions on sections.

Factors that may affect the evaluation of quantitative IHC data were listed but not necessarily placed in their order or importance. This would be difficult to do, as the relative importance of factors may differ for different antigens and histochemical procedures.

As far as can be determined, there appear to be no definitive articles that unequivocally show that IHC reactions are stoichiometric. In most laboratories, it is impractical to accurately determine section thickness, and furthermore many of the image analysis systems used may be somewhat rudimentary. Accurate determination of section thickness may therefore be somewhat of a moot point. One important point to note is that usually only a small number of sections are examined, and this often limits or prevents the generation of meaningful statistics.

In the USA, the increasing use of quantitation of IHC may be due, not to the need for greater reproducibility and accuracy, but to the need to justify the pathologist's decision in difficult (borderline) cases. It is questionable whether this can be accomplished to the degree that is always required – due to the limited number of sections customarily used and other factors.

Is the measurement of nuclear antigens useful? This is a difficult question to answer. The majority of papers in which nuclear antigens have been accurately quantitated have been electron microscopy rather than light microscopy based, and have used IHC prior to processing.

For many years the tendency has been (at the light microscopic level) to use the thinnest possible sections to obtain the optimal resolution and clear nuclear detail. This has increased the possibility of variation in section thickness and also the detachment and loss of portions of nuclei from the surrounding tissues. It should be noted that this problem of detachment can occur not only with nuclei but also with other structures including nonattached cells such as erythrocytes.

Standardization of fixation is difficult due to the large number of variables including prefixation time, amount of free blood, volume of fixing solution, size and composition of tissue, and so on. The routine fixation steps carried out in most pathology laboratories essentially result in only a partial fixation, and even with standard sized blocks of the same tissue there can be considerable variation.

There is no absolute answer as to whether quantitation is a useful tool in IHC. The question that should be asked is what significance will the pathologist place on the IHC quantitative data? Pathologists use their considerable expertise, and in most cases base their diagnosis on many factors, of which the IHC quantitation may play a minor or maior role depending on individual circumstances. ■