Quantitation In Immunohistochemistry

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Valuable information concerning the relative amounts of the end proucts of histochemical and immunochemical reactions present in sections may be provided by qualitative evaluations, however, greater reliance is often placed on quantitative evaluations. Many quantitative evaluations are based on the use of image analysis and optical density readings of the visible end products. An important question is whether these quantitative measurements are reliable, accurate and reproducible, and if quantitation of these reactions offers any real advantage over qualitative evaluations.

"Intensiy" and "optical density" are terms, which are often used to describe the amount of the end product of a reaction in a section, but these terms are often used in an undefined and indiscriminate manner. **In optics**, intensity of light refers to the amount of the light that is available to form the image. This measurement can be applied to both transmitted and reflected light microscopy and **is measured on a linear scale**. On the other hand, in reference to staining and histochemical reactions, intensity is used to **subjectively** describe the relative amount of the end product that is present (*e. g.*, - to ++++).

Optical density is the amount of light that passes through an object compared to the total amount of light incident to that object. Dark objects, which impede transmission of light, therefore, have a high optical density and lighter objects a lower optical density. **Optical density is measured on a logarithmic scale** from 1 (the lightest) to 4 (the darkest).

To illustrate some of the problems in measuring amounts of the end product, let us assume that tissues have been subjected to a **standardized** procedure of fixation in buffered formalin and paraffin wax processing. The histochemical procedure has resulted in a colored end product. Seven µm thick sections from different blocks of tissue have been measured on a video digital image analyzer. The optical density measurement from a section of the first block of tissue was 0.9 and from the second tissue was 1.0. As optical density is on a logarithmic scale, these measurements seem to indicate that, based on the limited number of sections examined, one tissue has twice the amount of material than the second.

In determining the reliability to be placed on this data, at the very least the following criteria should be applied:

1. Are the sections of the same thickness?
2. Was a standardized method of preparation used for each of the slides?
3. Were the measurements from comparable areas in different sections? Made by the same operator? Reproducible?
4. Do measurements correlate to the ratings the pathologist would give with a subjective or semi-quantitative method of evaluation? As a mini mum, are positive and negatives strictly comparable?
5. Were appropriate negative and positive controls used?
6. Does the counterstain obscure some of the weaker reactions?
7. Is the relationship between the color developed and the amount of substrate or antigen present known?
8. Can any artifacts present be identified?
9. Is the same image analysis system with the same settings used for all measurements?

In general, sequential sections cut from the same block at a particular setting are regarded as having plano-parallel upper and lower surfaces and to be of uniform thickness. There is, however, considerable compression during cutting and some non-uniform expansion between different tissue components during the section mounting. Compression of paraffin sections is dependent on a variety of factors including section thickness, size of the block, degree of hardness of tissue and its composition and many other technical factors. All other factors being equal, compression is usually inversely proportional to the section thickness. What is the thickness of the sections being measured? Direct measurement of section thickness is rarely carried out as this is cumbersome and time consuming; however if one section is 3 microns and the other is 4 microns then there is a 33% difference in the volume of tissue between these two sections. Careful measurements have shown that many sections are wedge shaped rather than plano-parallel. While variations in thickness between sections do not appear to be large, errors in measurement are usually cumulative and this discrepancy may be significant especially at higher magnifications.

Controls to decrease or eliminate these discrepancies can be incorporated. Controls can take a variety of forms, *e.g.*, a uniform block with a known concentration of protein such as gelatin or a relatively uniform tissue such as liver. Ideally, a block of this control material should be subjected to the **identical** procedures of fixation, processing, cutting, mounting, and staining as the tissue under investigation and also embedded in the **same block**. The reaction product in this standard control can then be directly related back to previous measurements. With careful planning this standard can also be the positive control.

Areas of the tissue, which do not appear to be stained, are routinely used as negative controls (background) to set a zero reading for the measurement of optical density. Significant differences can, however, exist between different "background" areas in the same section.

Operator measurement error can be significant and must be minimized and standardized. It is important to determine the error that is present for each individual operator and its repeatability. Initially, operator error can be determined by measuring single sections several times and calculating the standard error. It is usually not possible to produce identical readings, however, readings should be within well-defined limits. The acceptable degree of error will depend, to a large extent, on the differences being measured. The smaller the differences between samples, the more critical a low operator error becomes. As samples are often measured at different times, it is also important to determine reproducibility of measurements for the same sections at different times. It is critical that appropriate positive and negative controls be used.

The image analysis system should also be calibrated to reflect the expertise of the individual pathologist. Slides that can be easily ranked by a pathologist *e.g.*,on a scale of 1 through 5 should be measured and the readings compared to the pathologist's ranking levels. This will be a guide in setting the minimum and maximum levels of acceptance. If more than one pathologist is involved then each pathologist needs to be individually calibrated to the same standards.

A counterstain in a contrasting color may be useful for orientation but should not obscure weak positive reactions or be difficult or impossible to eliminate from the measurements. For orientation purposes, an alternate to the use of a counterstain is to consider a different type of microscopy such as darkfield, phase contrast, modulation contrast or interference microscopy to provide the additional contrast necessary.

Standardization of the reaction with attention to reagents, dilutions, expiration dates, times in reagents and the elapsed time before measurements are taken is important to varying degrees. There may be difficulties if the reaction relies on an individual technician to determine the final appropriate intensity of the end product. If either inadequate controls or no controls are used for calibration then additional problems will occur.

Several artifacts are possible during preparation of the stained section. For example, if the section contains areas with folds, undulations or bubbles, the reaction may have proceeded from both sides of the section, resulting in a greater intensity of staining in those areas. These areas may be in specific locations suggesting a greater amount of end product.

It is important to use the same image analysis system with the same illumination and filters to standardize measurements. If color is the major factor used in measurements then the color temperature of the illumination (and film if used) is an important factor. The illumination must be set at the appropriate level for this color temperature. If the intensity of light is too great for the image analysis input or film then this can be decreased using neutral density filters which will decrease intensity without altering the color temperature.

In addition to the above criteria, if photographic prints or slide transparencies are used as the image input they must be standardized. Daylight film can be substituted for tungsten film if the appropriate filter is used to adjust the color temperature. Color balance will, however, vary between film types from different manufacturers and even within batches of the same film, especially if stored at room temperature. For a more uniform result it is advisable to purchase a large batch of film and store in the refrigerator or the minus 20 freezer.

Many photographic processing companies adjust film processing to the color balance they consider appropriate and this is more apparent with color print than color slide film. Standardization requires that a standard sample of a color print or color slide, as applicable, be supplied each time a film is submitted to ensure that the photographic processing is uniform from film to film.

The above considerations do not necessarily take into account other factors that are important when using fluorescent markers, *in situ* hybridization and autoradiography. Fluorescent markers have additional considerations of fading, standardization of reagents and procedures.

Procedures which measure exposed photographic emulsion require the use of standards such as aluminum step wedges or sections of known radioisotope content on the same slide. Controls on separate slides will create additional errors.

With the above points in mind the question is, "Do optical density measurements provide useful quantitative data from slides stained for immunohistochemistry or histochemistry when compared to subjective (qualitative) evaluations? At first glance the comments above might suggest that we are dealing with an insurmountable problem with so many potential errors that not all can be recognized, eliminated or standardized. The keys to successful measurements are standardization and controls.

An image analysis system, if correctly calibrated, operated by a skilled technician and using appropriate controls, can provide quantitative data that is both consistent and meaningful. To reach that point however requires expense, training and meticulous attention to detail. The critical point in the entire process is the definition by the pathologist of clear parameters, including examples of positive and negative cases, to ensure that measurements within a specific range level reflect the pathologist's perception of the diagnoses. In cases, which are unequivocally positive or negative, measurements may offer no real advantage to the pathologist. In other instances, which could be classified as marginal, the data may be a deciding factor, providing that the appropriate levels of acceptance have been clearly defined.

The skill of an experienced pathologist will never be replaced by an image analyzer, however, the quantitative data provided by such systems may aid in the clarification of difficult cases and enable more specimens to be examined in a shorter time period.

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