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EDITORIAL

New quantitative data on cell blocks

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The goal of cytology is to provide a diagnosis with the smallest possible sample. A major challenge is to include the increasing number of essential ancillary tests into the diagnosis.[1,2] How small can a sample be and still provide all the diagnostic information that may be needed? We know that 5000 cells contain about 35 ng of DNA, more than needed for some next generation sequencing methods.[3] If we assume that an additional 500 cells are needed for a morphologic diagnosis, and 500 cells are needed for each of 15 immunostains that may be required in an exceptionally complicated case, we only need about 13,000 tumor cells. If a sample were to contain 10% tumor cells, and if each cell measured 20 microns in diameter, the 130,000 total cells would have a total volume of only about 0.5 μL (for reference, one drop is typically about 50 μL). If centrifuged, the pellet of 130,000 total cells would likely be invisible. The number of needed cells could decrease further with new technologies such as the Phenoptics multiplexed immunofluorescence platform by PerkinElmer (Waltham, MA) that can allow multiple simultaneous immunofluorescent stains on one section (retrieved from http://www.perkinelmer.com/product/single-multiplex-immunohistochemistry-ihc-immunofluorescence-if-ihc-svc).

A tiny fraction of a drop is not a big sample, but in fact many studies suggest that cytology specimens, and even small histologic biopsies—which should be orders of magnitude larger than needed—are commonly inadequate for testing due to insufficient cells.[2,4,5] It appears that the biggest obstacle to using the smallest possible biopsy is the technical challenge of handling and processing a potentially invisible, yet diagnostic, sample.

Many laboratories successfully use cell blocks for the ancillary testing,[6,7] but some laboratories express dissatisfaction with their cell blocks[8] and have had to adapt and validate smears for ancillary molecular testing,[9,10] or shift away from cytology toward use of core biopsies. What factors account for the variable results with cell blocks when we try to approach a goal of using a minimal sample?

The new article by Torous, Chen, and Vanderlaan (in this issue of JASC) provides important new quantitative insights that help expose 2 key variables. Quantitative information on cell-block performance is surprisingly limited.[11] The limitation has to do with the difficulty in quantifying the input material and the difficulty in quantifying the number of cells per cell block histologic section. There is also a difficulty obtaining replicates for study: The heterogeneous nature of cytologic samples makes it difficult to divide them equally. In addition, artificial cytology samples prepared from resected tissues do not emulate samples acquired from a living patient (eg, due to the absence of blood). Torous et al chose to study a relatively quantifiable endpoint (the number and size of granulomas) in a homogeneous sample type: real-life endobronchial ultrasound samples. The authors also cleverly corrected for sample-to-sample heterogeneity by comparing the numbers and sizes of granulomas on the ThinPrep to a cell block section from the same case. Three types of cell blocks were compared: plasma thrombin, HistoGel (ThermoFisher Scientific, Waltham, MA), and the new cell-gel[12] blocks. They found that cell-gel cell blocks display significantly more granulomas than HistoGel cell blocks, and somewhat more granulomas than plasma thrombin cell blocks.

The authors attribute the better performance of cell-gel blocks to the increased surface area of the solidified gel.

Key point 1: To maximize the number of cells displayed in a cell block section, distribute the sample into a 2-dimensional plane.

It stands to reason that at a given concentration of particles/cells in the paraffin, more will be seen if the 2-dimensional area containing the cells increases. In other words, spreading the material into a 2-dimensional plane with a larger area should proportionally increase the amount displayed in a paraffin section. The HistoGel and plasma

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thrombin methods usually rely on using the conical base of a 50-mL centrifuge tube as a mold for the coagulated cell block (though larger bases are available[13]). The conical base of the standard 50-mL tube has a diameter between 5 mm and 12 mm in the bottom 5 mm of the tube, which would have an area of about 50 mm^2 in a paraffin section. In comparison, the cell-gel method uses a 15 × 15 mm square base that would have an area of 225 mm^2. All things being equal, one would expect 4.5 times more granulomas to be displayed in the cell-gel blocks that distribute fragments into a larger surface area. In fact, Torous et al did find a higher recovery in cell-gel blocks compared with HistoGel.

What other considerations could affect yield? As previously pointed out,[14] it also stands to reason that the number of cells per volume within the paraffin affects the number of cells per 4-micron section: dilution of cells with agar, fibrin, or other extraneous material will necessarily decrease the number of cells that can be seen per section.

Key point 2: Minimize the amount of extraneous noncellular material in order to maximize the number of cells displayed in a cell block section

In the carefully documented study by Torous et al, HistoGel blocks were produced with 10 drops of gel (about 500 μL agarose). We know that 500 μL agarose would make the block have a thickness of at least 5 mm. A 4-micron section of such a block would display a mere 0.08% of the sample. A 5-mm thick suspension of cells would be useful if you needed 1250 histologic sections, but it is rare to ever need more than about 20 sections. The authors note that they used only 6 drops of plasma thrombin for the cell blocks, which should make a sample appear to have about 1.7 times more granulomas per section compared with using 10 drops of HistoGel. In fact their plasma thrombin cell blocks had an average of 2.3 times more granulomas compared with HistoGel.

Key point 2 can be hard to appreciate. Many factors can cause a sample to be lost through dilution, factors that create a kind of needle in a haystack problem. Some factors are unavoidable (eg, abundant cystic debris, necrotic material, unclotted and especially clotted blood). Importantly, however, many of the factors that dilute a sample are self-inflicted. Agarose and added fibrin both dilute the sample such that fewer cells are seen per section. The dilution of a sample by HistoGel can be countered by centrifuging the sample before it congeals, thereby concentrating the sample in 1 plane.[13] Other less-obvious factors that result in a diluted cell block include use of formalin or other fixatives that precipitate non-cellular proteinaceous debris. Formalin is a particularly poor needle rinse solution for cytology because it precipitates abundant acellular debris. For example, if 100 μL whole anticoagulated blood (2 drops, emulating a typical fine-needle aspiration sample, containing only about 50 μL packed red blood cells), is suspended in 10 mL formalin, incubated for 30 minutes, and then centrifuged, the volume of the pellet (containing unlysed, fixed red blood cells and precipitated serum proteins) measures close to 100 μL (Andrew Fischer, unpublished findings). If the same experiment is conducted with CytoRich (supplied by BD, Franklin Lakes, NJ), all the red blood cells are lysed, their protein and the serum proteins remain solubilized, and the pellet is essentially invisible (though it contains about 500,000 fixed white blood cells). It is much easier to find the needles when the haystack is not there. The only reason to use formalin in the cytology laboratory is to avoid the effort and expense of performing validations.[3]

The authors also suggest that lower yield in HistoGel blocks compared with cell-gel blocks could be due to difficulty removing the entire sample from the conical tube, but it may actually be easier to remove a coherent plug of cells that are trapped in HistoGel rather than trying to scoop out a loose pellet of cells and manually transfering them to the 15 × 15 mm mold. Molten HistoGel could be used to rinse out all the particles from the tube for a cell-gel block, but this has to be done quickly and would likely require some visual feedback to optimize efficiency. To rely on visual feedback requires a relatively massive sample, and, ideally, cell processing techniques should be able to be performed without requiring visualization of the sample.

Can these 2 key points and the consideration of visual feedback help us predict the success of other cell block techniques? Collodion bag cell blocks are highly praised by many,[15] yet the collodion bag process does not arrange cells into a 2-dimensional plane. It seems likely that the success of collodion cell blocks may be because they do not dilute the sample with any additional agar or fibrin. In addition, except for potential losses decanting the supernatant, the entire sample is recovered, even if it is invisible. Cellient (Hologic, Marlborough, MA) automatically embeds cells and fragments in a plane 12 mm in diameter (113 mm^2), within about 30 minutes, without the need for any extraneous diluting substances. The cellular plane is marked with eosin and the plane is indexed to a microtome cassette, helping the histologist find the plane containing the cells. Visual feedback for embedding is not needed and complete recovery is possible. Cellient is compatible with any fixation (including formalin), but in our experience, Cellient works optimally when a sample is collected in CytoRich red to avoid precipitating any non-diagnostic debris. The principal limitations of Cellient include its cost and the potential need for validations if formalin is not used.

Beyond the insights into cell block performance, an interesting finding by Torous et al was that the size of the granulomas were distinctly smaller in ThinPrep slides compared with the matched cell-block sections. A 5 micron section can transect the granuloma near its edge, such that the largest diameter may be rarely displayed. However, the unsectioned granulomas presented in a ThinPrep slide must show the largest diameter. The authors thus convincingly demonstrate that the ThinPrep process tends to recover the smaller fragments in a heterogeneous mixture. This finding justifies the practice in their lab (and ours) of making

a ThinPrep first, and then making a cell block on the relatively large fragments that are left over. A final very important quantification by Torous et al provides a justification for making cell blocks: In 19% of cases, granulomas were only evident in the cell block sections, not the ThinPrep slide.

Quantitative data are important in solving the technical challenges that lie between us and our goal of using the smallest possible biopsy. It will be important for new publications to be described with the degree of quantified detail provided by Torous et al to help us reach this goal.

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