Scan-and-treat methodology using Azure A fast stain as a cost-effective cervical cancer screening alternative to visual inspection with acetic acid

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Introduction

Most cases of invasive cervical cancer arise in the developing world, where few resources exist for large-scale screening programs. Sankaranarayanan [1–5] has advanced visual inspection with acetic acid (VIA) as a method to screen women in low resource settings. While VIA has been extensively investigated, most studies rely on a gold standard of colposcopically directed biopsy. In our studies [6], the sensitivity of colposcopy is 96% and the specificity is 48% when discriminating between normal and abnormal cervical tissue. Other studies, such as the ALTS [7], report sensitivities for colposcopy ranging from 50 to 85%. Because abnormalities are missed by expert colposcopists, the promising results of VIA trials must be qualified by the fallibility of the criterion standard. False negative results are therefore troublesome for VIA and colposcopy.

The best validated screening tool for cervical cancer is the Papanicolaou smear, which has been demonstrated to lower morbidity and mortality from cervical cancer over the last 40–50 years in the United States, Canada, the United Kingdom, and Scandinavia. Our group has studied the Cytosavant, an automated computer assisted system for measuring quantitative properties in the cell. These measures include: ploidy, texture features, and nuclear architecture [8]. Pathology slides are read automatically using a pneumatic system for placing the slide on the microscope and taking it off the microscope. The system typically uses a Feulgen stain that is stoichiometrically related to the amount of DNA in the cell. The system reads 2000 cells per slide and gives a summary diagnosis. This system is in use in China, where our collaborators report a processing cost of approximately $3 USD per slide [9].

Automated quantitative pathology using a system like the Cytosavant could be an optimal solution for large-scale screening programs in the developing world and in underserved areas of North America and Western Europe. The principal disadvantage of the system is that patients currently would have to wait overnight to receive a reading. In low-resource areas, many of the major barriers to effective screening involve both cultural and transportation issues that make it difficult to bring patients to the clinic in the first place [10]. The necessity for follow-up visits magnifies these problems. In order to optimize an automated quantitative pathology system for deployment in

Table 1

Protocol comparison between Azure A and thionin stain procedure

<table>
<thead>
<tr>
<th>Azure A stain procedure</th>
<th>Thionin stain procedure</th>
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<tbody>
<tr>
<td>Rehydration through a series of alcohol steps (100%, 75%, 50%)</td>
<td>Boehringer fixative at 26 °C for 45 min followed by a water rinse</td>
</tr>
<tr>
<td>5 N HCl acid at x°C for 9 min</td>
<td>5 N HCl acid for at 26 °C 60 min followed by a water rinse</td>
</tr>
<tr>
<td>Azure A solution at x°C for 10 min</td>
<td>Thionin stain at 26 °C for 75 min followed by a water rinse</td>
</tr>
<tr>
<td>1% acetic acid for 2 min</td>
<td>3 changes of rinse reagent (30 s, 30 s, 5 min) followed by a water rinse</td>
</tr>
<tr>
<td>Dehydration through a series of alcohol steps (50%, 75%, 100%)</td>
<td>Clear with xylene</td>
</tr>
<tr>
<td>Clear with xylene</td>
<td>Clear with xylene</td>
</tr>
<tr>
<td>Mount slides</td>
<td>Mount slides</td>
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</table>
the developing world, the time between the clinical visit and completion of the specimen analysis would have to be shortened to a time span of several hours.

To address this issue, we have begun development of a fast stain that can be done in 2 h so that the patient could be given a result the same day the smear is taken. This would permit patients to be treated on site, if necessary. By eliminating the need for one follow-up visit in the case of an abnormal smear, a principal barrier to screening in low-infrastructure regions can be circumvented. Moreover, since the reading is automated, health care workers could be trained to perform Papanicolau smears without acquiring the considerable training and experience required to most reliably perform visual inspection.

Methods

The fast stain process under development uses an Azure A solution for nuclear staining of liquid-based cervical specimens. The specimen undergoes a process of graduated hydration prior to staining and graduated dehydration after staining and prior to clearing and mounting. This is in contrast to our current procedure for thionin staining, in which samples are not hydrated prior to staining and the dehydration alcohol is undiluted. For a summary comparison of the Azure A and thionin stain procedures, see Table 1. The thionin stain has been

Table 2
Time comparison between Azure A and Feulgen-Thionin stain procedure

<table>
<thead>
<tr>
<th></th>
<th>Azure A</th>
<th>Feulgen-Thionin</th>
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<tbody>
<tr>
<td>Reagent Preparation</td>
<td>~10 min</td>
<td>1 hour and 30 min</td>
</tr>
<tr>
<td>Stain reusability</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Stain time only</td>
<td>~1.5 hours (30 min for staining + initial 1 hour to warm water bath)</td>
<td>4.5 hours (3.5 hours for staining + initial 1 hour to warm water bath)</td>
</tr>
<tr>
<td>Total Time</td>
<td>~2 hours</td>
<td>~6 hours</td>
</tr>
</tbody>
</table>

Fig. 1. The “scan-and-treat” approach.
widely used by many research groups to quantify the amount of DNA in a tissue sample using image analysis systems [11,12].

Time is saved during pretreatment (9 versus 60 min), staining (10 versus 75 min), and rinsing (2 versus 5 min). Table 2 shows the total differences in time requirements for the Azure A and a Feulgen–thionin stain procedure. These time savings can make it practical to acquire specimens in the morning and complete analysis in time to perform treatment where necessary in the afternoon. For a diagram of this ‘scan-and-treat’ approach, see Fig. 1.

Results

In Fig. 2A, the area of the nucleus of cervical cells stained with Azure A at 30 °C is plotted against the DNA index. A DNA index of 1 represents a normal cell, while a DNA index of 2 is a tetraploid cell (a dividing cell). In Fig. 2B, we show that at a temperature of 30 °C, the amount of DNA noted is similar to that shown by the Feulgen stain, which is routinely used at 26 °C. As we are trying to develop a comparable stain, we believe that the 30 °C condition is the ideal temperature for use in this screening model.

The coefficient of variation (CV) is an important parameter of device performance and biologic variability. Previous studies have revealed the CV for the Feulgen stain to range from 4 to 5% [8]. In Figs. 3A and B, we plot coefficients of variation against the staining procedure (Feulgen or Azure A at varying temperatures). For diploid cells, the CV is 6–7% for the Azure A stain at 30 °C. For tetraploid cells, the CV is between 5 and 6%. While it is preferable to have as low a CV as possible, we believe that 5–7% is acceptable and will improve as we refine the procedure.

We are able to model the effects on test characteristics (sensitivity and specificity) of coefficients of variation ranging from 0 to 8%. These measures are impacted by the quantity of aneuploid cells that is used as the threshold for positivity. In Fig. 4, it is shown that test characteristics vary with threshold selection and coefficient of variation.

The Azure A stain shows a coefficient of variability (CV) of 5–6%, compared to 3–4% for a Feulgen stain. The relatively increased variability should not present a problem for a DNA ploidy analysis because the same sensitivity and specificity should be achievable by adjusting the threshold DNA index. While an increased CV tends to lower specificity, while increasing sensitivity, the effects are not dramatic when CV = 5–6%. Fig. 4 shows how sensitivity and specificity are mediated by variability and selected DNA index thresholds. CV must be 5–6%, it is even in early studies.

While we continue to refine the staining protocol, we will report on efforts to model the effectiveness of the fast stain in a

![Fig. 2. (A) Azure A — temperature 30 °C. (B) Raw DNA amount of diploid cells.](image)

![Fig. 3. (A) Diploid cells DNA index CV. (B) Tetraploid cells DNA index CV.](image)
screening program based on automated cytology. A future manuscript will detail the effect of increased staining variability on the DNA content histogram.

Conclusion

The fast stain procedure is an emerging technology that is tailored to the end user and optimized for deployment in low-infrastructure settings, where the need for effective screening programs is most critical. We hope to continue to evaluate the effectiveness and plausibility of this technology, eventually by pairing a scan-and-treat approach with an upcoming screening program in Nigeria. Among screening patients in North America, the time lag between testing and receipt of results is a primary concern impacting overall patient satisfaction [13]. Because of this dynamic, further refinement of a scan-and-treat approach may also improve screening attendance and follow-up behavior in the developed world.

Conflict of interest statement

We declare that we have no conflict of interest.

References