Analysis of acetic acid-induced whitening of high-grade squamous intraepithelial lesions

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1 Introduction

Colposcopy is the first diagnostic test for invasive cervical cancer in women whose screening Pap test is abnormal. The exam is performed with high magnification, a bright light source, and acetic acid. Cervical patterns are interpreted by clinically standardized criteria. The colposcopic examination determines the location of the most severe dysplastic region for biopsy placement. A fundamental part of the colposcopic exam is the use of acetic acid, which when applied to the cervix induces transient whitening changes in the epithelial tissues. The spatial and temporal changes of acetowhitenning are the major visual diagnostic indicators in the examination, and are interpreted by the trained colposcopist based upon prior experience with the procedure. While this exam is an important diagnostic method, it suffers from being a subjective test because the kinetics used to diagnose cervical lesions are learned through experience and are subject to variation between physicians. Mitchell et al. reported that the sensitivity and specificity of the colposcopic diagnosis of high-grade lesions from all others was 86% and 69%, respectively, based upon meta-analysis survey of published data. The accuracy of colposcopy is a function of physician training, suggesting that interphysician variation in diagnosis will be present. There is increasing evidence that the colposcopic method would benefit from semiautomated algorithms which provide the physician with numeric scores for staging cervical lesions.

The opportunity to provide more objective assessment methods for cervical cancer diagnosis has motivated research into alternative diagnostic measurements such as fluorescence, reflectance probes, near-infrared hemoglobin probes, and image processing algorithms, which are more conducive to quantitative decision making criteria. The ideal goal would be to find numeric measures which can be quantitatively correlated to lesion stage, and thereby have higher predictive value. A logical step in this development is to identify measurements that are derived from the standard colposcopic examination to be used as a physician’s aid in cervical diagnosis. While there has been some study of the acetowhitenning phenomenon in high-grade lesions, there is limited data available in published form to develop metrics for classification. In this study, we examine the process of acetowhitenning measured by a standard digital colposcope and outline methods to quantify the kinetics of acetowhitenning in high-grade squamous intraepithelial neoplasia.

The temporal kinetic of the acetowhitenning process as measured by the reflected light maximizes within the first 1–2 min and decays over several minutes thereafter, allowing a subjective interpretation by the physician in real time. Digital colposcopy provides individual electronic pictures of the cer-
vix, usually taken at maximal acetowhitening, that have been used for patient visit documentation. By taking a series of images rapidly during the routine examination there is the opportunity to analyze the spatial and temporal changes with automated algorithms. The technology to achieve this image stream is simple, yet the image processing to accurately and quantitatively assess changes within the images is more challenging. In earlier work it was shown that there was likely more information about the dysplastic tissues in the blue-green region of the reflected spectrum. This was thought to be due to the fact that the tissue whitening process is a product of the relative overall increase in reflectance which occurs over all optical wavelengths, and diminishes the blue-green absorption of the blood which is present within the tissue. In this study this change in reflected spectrum is examined as a function of the time after application of acetic acid, allowing investigation of the spectral-temporal parameters which may be exploited for quantifying lesion stage. It is likely that some weighted combination of spatial, spectral, and temporal measurements will be needed to robustly characterize lesion stage, based upon the visual metrics which are routinely applied in colposcopy examination.

In this study we present the data from a series of patients with biopsy confirmed CIN 2/3 and those with normal mature epithelium. The system characteristics are examined, methods to acquire numerical data from the images are shown and some data normalization procedures are presented.

2 Materials and Methods

2.1 Patient Examination

All patients recruited into the study had index Pap smears which indicated squamous intraepithelial lesions or atypical squamous cells of undetermined significance. A total of 11 patients had biopsy confirmed CIN 2/3, but five of those patient data sets were eliminated due to lack of visualization of the disease region or due to incomplete data acquisition. The data sets from the six remaining patients with CIN 2/3 were used for this study. All patients underwent a standard colposcopic examination with automated time sequenced digital image capture. A timed image capture process was initiated before applying acetic acid to the cervix of each patient. Following standard procedures for colposcopic examination a solution of 5% acetic acid was applied to the cervical tissue surface for approximately 15–30 s. Each patient was followed every 20 s for up to 10 min total to allow capture of the decay from whitening. The physician (DMH) electronically marked the biopsy sites immediately after the exam, and denoted them on the printed color images at a later time. Biopsies were all processed according to standard clinical pathology procedures. Regions within each series of images were correlated to the gold standard histologic biopsy results. This allowed a prospective analysis of the tissue regions of interest (ROI), based upon the sites of biopsy. The Committee for the Protection of Human Subjects at Dartmouth College approved this study.

2.2 Colposcopy Imaging System

The colposcope was a standard Carl Zeiss system, which was fitted with digital imaging capabilities, with a Dage 3CCD color video camera, and standard Zeiss optics. The exposure time and sequencing of the images were automated by a custom written software package (Solutions TeleComputing, Hanover, NH). The light was delivered from a standard arc lamp assembly built into the scope. The light source used for standard colposcopy exams is a 150 W tungsten lamp, but the light source used for image capturing here was a 300 W xenon arc lamp which provided greater intensity to increase the reflected signal, thereby providing higher signal to noise ratio (Aurora Optics, Inc., Hanover, NH).

The imaging program acquired approximately one image every 20 s for up to 10 continuous minutes generating approximately 30 white light images. The images were saved as lossless JPEG format within the same program. The typical image noise level and spatial variation of the imaging field were determined through the use of a 10% reflectance standard, which approximated the reflectivity of cervical tissue.

2.3 Image Analysis

A separate person (A.Z.) who was given the images showing biopsy location and results completed all data analysis. A custom written image analysis program (MediAnal, Solutions TeleComputing, Hanover, NH) was used to process the data from each image sequence in a semiautomated manner. The locations of a region of interest (ROI) were sequentially placed on successive images, and could be translated under user guidance on any given image to correct for small patient movements. For each ROI corresponding to a biopsy, five small regions, each 5 pixels by 5 pixels, were sampled and then averaged together to reduce the variance in the temporal sequence of data. The MediAnal processing algorithm calculated the luminosity of the white light, as well as the red, green, and blue channels. The standard deviations over the regions were also calculated.

Patient movement, detector movement or source variation can cause intensity variations, making data normalization necessary to compare different patients’ sets of images. Since it is known that the reflectance change from tissue is dominant in the blue-green region, it is possible to separate out the color channels from the CCD measurement and ratio these channels. In our earlier study, we documented that most epithelial structures which were indicative of neoplasia were strongest in the blue-green spectral regions and that the red reflectance contained the least structure. Analysis of our earlier data suggested that the blue channel would provide optimal contrast between tissues that whiten and those that do not, however this issue is also convolved with which channels provide the best signal to noise ratio. Thus, we examined both blue to red ratio and green to red ratio as a method to self-normalize the data.

3 Results

3.1 Background Noise and Linearity Testing

Detected background noise with the room lights and imaging light source off were typically 12–13 counts (±2 SD) and a maximal spatial variation of four counts. When a 10% reflectance...
tive target was used as the test object, a signal of 100 counts
(± 4 SD) was measured. The signal varied spatially, as with
all imaging systems of this type, with a 35% maximal de-
crease laterally from the center to the edge, and a 20% de-
crease vertically from center to edge. Longitudinal translation
of the target towards or away from the focus of the imaging
system resulted in a 0.3% per mm change in detected inten-
sity. Examining the individual data from the three color chan-
nels was the subject of part of this study, and while there is
considerable interpatient variation in the remitted light, in
general the standard deviations of the three channels was
4.8%, 3.8%, and 6.3%, for red, green, and blue, respectively,
for a nonmoving target.

3.2 Patient Motion and Correction
Specific tissue sites were marked on each image for all six
patients to determine the effects of patient movement. While
on average the patient motion was typically less than 1 mm,
maximal average changes in site position for these patients
were 4.5 mm. In all image sequences analyzed, the ROI po-
sitions were validated manually to be in the same location
using a semiautomated procedure that was built into the soft-
ware.

3.2 Patient Data
The data from patients with biopsy confirmed CIN 2/3 were
included in this study. The quality of a typical set of electronic
images and the typical aceto-whitening levels observed in a
cervix with significant areas of CIN 2/3 is illustrated in Figure
1. In this case, much of the transformation zone around the
cervical os contains CIN 2/3. In the first displayed image
taken immediately after the application of acetic acid, pooling
of the acetic acid in the bottom of the image caused the lower
half of the cervix to be blurred. In the second image, taken 20
s later, the squamous epithelial lesion has whitened signifi-
cantly, and continues to whiten for the next two images. The
final two images illustrate the decay of aceto-whitening which
occurs on the timescale of several minutes, ultimately decay-
ing back to background levels in approximately 10 min.

The data are measured from six women with two epithelial
types: CIN 2/3 and mature squamous epithelium. For each
tissue type, CIN 2/3 and the mature squamous epithelium, the
data were normalized to have an average value of 1.0. The
intensity of the CIN 2/3 region doubles during the first minute
after acetic acid application. In contrast, the region of mature
squamous epithelium increases maximally by 5% over the
entire time sequence of 10 min.

3.3 Normalization vs Ratio Data
We examined both blue to red ratio, green to red ratio, and
green to blue ratio as a method to self-normalize the data
within an individual temporal stream of data and remove in-
tensity shifts which may be more dependent upon light col-
collection efficiency rather than due to intrinsic tissue character-
istics. The same ROI data were used in Figure 2(a), and the
resulting ratio data are shown in Figures 2(b)–2(d), for both
the CIN 2/3 and mature epithelium case. These ratios appear
to reduce some of the noise in the raw intensity data, which
presumably comes from patient movements during the exam.
While the graph in (b) appears to have the greatest contrast in
CIN 2/3 signal between peak and background, it suffers from
higher noise than the data in (c), where the green to red ratio
was used. This is most obvious in the mature epithelium data
where the scatter is significantly reduced in the ratio values
(c) as compared to the absolute values (b).

The data from the six patients with CIN 2/3 and the six
patients with normal mature epithelium were plotted with the
green to red ratio, and the traces were normalized such that
the maximal value was unity for each temporal data set. These
data are plotted in Figure 3. The data were further processed
to average the intensity for each 100 s interval, which simply
provides a time-averaged smoothing to the data, and reduces
any high frequency oscillations which are likely due to patient
movement. These averaged data are plotted in Figure 4. Using
this data set, the curves of CIN 2/3 and normal mature squa-
mous epithelium are significantly different in shape, and all
time-paired points for normal and CIN 2/3 tissue plotted in
Figure 4 are statistically different from each other (P < 0.05)
based upon a students t test.

3.4 Data Reduction
In order to provide a single number from the temporal se-
quence observed from each patient, the slope of the reflected
intensity versus time was examined as a predictive variable.
From the data in Figures 1 through 3, it can be seen that CIN
2/3 regions are characterized by a rapid increase in reflectance
within the first 60 s, followed by a large decrease. In most
cases, the initial data before and immediately following the
application of acetic acid could not be captured here due to
practical problems of the physicians procedure blocking the
view of the camera to the cervix. Nonetheless, the reflectance
data at times beyond 20 s after the application of acetic acid
was readily captured, and the transient behavior was distinctly
different for normal tissues. In contrast to the CIN regions,
the mature epithelium showed a moderate increase continuing
for up to 300 s, followed by an almost constant reflectance over
the time of acquisition here. To quantitatively compare these
observations, the slopes of intensity versus time were calcu-
lated for each patient sequence shown in Figure 3. The time
sequence was dichotomized to before 200 s and 200 or more
seconds. The slopes are plotted against one another on param-
etric graphs in Figure 5(a). This plot provides separation of
the two tissue types into the upper right quadrant for normal
tissue and lower right quadrant for CIN 2/3.

Another method to separate the two tissues is to simply
compare the time averaged intensities which are displayed in
Figure 4. An illustration of this is shown in Figure 5(b) where
the average intensity in the first 100 s is plotted against the
average intensity of the values between 200 and 300 s. This
method of categorizing the data provides even clearer separa-
tion than the slope method for these two tissue types. The
normal mature squamous epithelium data appear along the
horizontal axis and the CIN 2/3 data along the vertical axis.
The ratio of the long time data (200–300 s) relative to the short
time data (100 s) was calculated for these points, and
provides a statistically significant method to separate the data
which is independent of any data normalization. In this data
set, the mature squamous epithelial tissue regions have an
average ratio of 1.04±0.04, and the CIN 2/3 regions have a
value of 0.85±0.06. A students t test of these data sets indi-
cates that they are significantly different with a $p$ value of less than 0.0001.

4 Discussion
The major accomplishment of this study has been to accumulate reflectance intensity data for up to 10 min from patients with biopsy confirmed CIN 2/3. There is a 10%–50% change in median values of normalized intensity after the application of acetic acid, as shown in Figure 3(a). The visual changes of acetowhiteness disappear from CIN 2/3 tissues rapidly, while the decaying reflectance values from mature squamous epithelium remain moderately elevated for the entire 10 min, as shown in Figure 3(b). The variation among patients with CIN 2/3 in intensity of acetowhiteness is significant, and the time constant can vary significantly even within a single lesion, as observed from the images in Figure 1. The initial rise in tissue

![Fig. 1 Time sequence of images from a cervix with biopsy confirmed CIN 2/3 in the left lateral region from the cervical os, marked with an arrow in the second image. The images were taken (a) immediately after application of acetic acid, (b) 20 s after, (c) 60 s after, (d) 80 s after, (e) 6 min after, and (f) 9.5 min after.](image-url)
whiteness in the CIN 2/3 tissues is rapid, and technical difficulties of applying the acetic acid to the cervix and allowing the residual to drain away prohibit an accurate measure of this initial rise in reflectance. Note that the data from the initial 10–20 s was not reported in Figure 3, because the presence of acetic acid blurred the images during this time. Rough estimates indicate that this rise is anywhere between 20% and 80% of the original reflectance intensity, but measurement during this time period is logistically difficult to achieve. Future studies may examine better experimental designs to allow quantification of this initial rise time.

In contrast, mature squamous epithelium appears to increase in reflectance by approximately 5% on average with a standard error of nearly 5%, and tended to remain raised for the duration of our 10 min imaging period. Individual patient tissues vary considerably, and without some method of data normalization it would be difficult to quantitatively compare the results between patients with sufficient predictive value. Patient movement during the course of the imaging session was the most problematic issue for automating the data acquisition system, and ultimately a manual correction had to be applied to our image analysis to help maintain a good spatial correlation between the site of biopsy and the ROI chosen on the image by the clinician. Future studies may be able to improve this approach to the data analysis by implementation of motion reduction processing in the software or hardware.

Normalization of the data to the average value can provide a good first attempt to remove interpatient variation in reflectance.

Fig. 2 (a) Reflectance intensity as a function of time after application of acetic acid is plotted from a ROI in one patient. The ROIs are a biopsy confirmed cervical intraepithelial neoplasia (CIN) 2/3 region and a normal mature squamous epithelium (MSE) region. The data values were normalized to the average intensity of the entire time series. In (b) the ratio of blue to red intensity is displayed for the same data, in (c) the ratio of green to red is displayed, and in (d) the ratio of blue to green.

Fig. 3 Plots of normalized green/red intensity ratio as a function of time after the application of acetic acid, for (a) the six CIN 2/3 cases and (b) the six mature squamous epithelium cases.
distance patterns. The intensity ratio of green to red light provides the best tradeoff between signal to noise and contrast, as a way to normalize the transient data within each picture in the time sequence. Normalizing the green by the red light preserves kinetics in reflected signal, indicating that the reflected light has a spectral change. While comparing initial and final slopes can provide a good method for tissue classification, using the simpler time-averaged intensities appears to be a more robust method. We suggest that taking the ratio of short to longer time points for this green to red ratio signal provides an accurate way to separate out different tissue types. In this study, the ratio of the green/red reflectance signal at 200–300 s relative to 100 s provides a statistically accurate method to separate CIN 2/3 from mature squamous epithelia.

It is important to note that the reflectance change is thought to result from index of refraction changes in the cell nucleus.\(^{21,22}\) It is known that this subtle change in cellular organelles can be characterized by using the full spectrum of white light and fitting the spectrum to predictions from Mie scattering theory.\(^{23}\) A more detailed analysis of the spectral features of this acetowhitenign may reveal discriminatory features such as those used by Backman et al.\(^{24}\) to classify dysplastic versus normal tissues in the esophagus, colon, bladder, and oral cavity.

Ultimately a classification algorithm will have to make use of all the pertinent measures that colposcopy physicians currently use in their analysis, including morphologic feature detection as a function of time. In our previous study\(^{15}\) we illustrated that the Euler number provided a good metric for classifying CIN 2/3 tissues, and it is likely that by examination of this measure in the temporal sequence improved classification will result. Combination of morphological detection algorithms together with these temporal change algorithms are needed to provide measures of as many pertinent colposcopy parameters as is possible from this type of digital information. Future studies in this direction are needed to provide semi-automated algorithms for staging cervical lesions including low-grade intraepithelial lesions (LSIL/CIN 1/HPV), and metaplasia. This comparison is ongoing at present and will be the subject of a future study.

5 Conclusions

In conclusion, this study presents the time sequence data of CIN 2/3 and normal mature squamous epithelium acetowhitenning. By using the ratio of green to red light from the color images of the cervix, a self-normalized data set can be developed which minimizes artifacts due to patient motion and reflected intensity fluctuation. The increase and resulting decay in reflected intensity can be 50% in this normalized green to red ratio for CIN 2/3 on average. The increase in reflected intensity in normal mature epithelium is near 5% on average. Comparing the normalized green to red ratio where the data are time averaged over 100 s intervals provided a robust method to distinguish mature squamous epithelium from CIN 2/3 in this data set. This report is a feasibility study which may provide a method to separate cervical lesion regions from normal regions based upon relatively low-technology tools which can be used in a standard colposcopic imaging session.

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References

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