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# Real-Time, High-Definition, Three-Dimensional Microscopy for Evaluating Problematic Cervical Papanicolaou Smears Classified as Atypical Squamous Cells of Undetermined Significance

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**BACKGROUND.** The perceived inadequacies of the cervical Papanicolaou (Pap) smear have been attributed to sampling, screening, or interpretive errors. Within this type of cytologic preparation, there are thick cell clusters in which the cells are *obscured*. It may not possible to evaluate these areas by conventional microscopy. The authors clinically tested the hypothesis that high-definition, three-dimensional (3-D) microscopy based on multiple oblique illumination (MOI), with its ability to penetrate into thick areas, would be useful in evaluating problematic cervical Pap smears, particularly those diagnosed as atypical squamous cells of undetermined significance (ASCUS).

**METHODS.** ASCUS Pap smears and corresponding surgical biopsy specimens were evaluated prospectively using standard, axially illuminated microscopes and a new high-definition, 3-D microscope employing MOI. The Pap smears were reviewed in a blinded fashion with both types of microscopy. The rendered diagnoses were then compared with the subsequent tissue biopsies, which also were blinded, as the definitive end point.

**RESULTS.** It was immediately apparent that the high-definition, 3-D MOI microscope had better resolution compared with the standard microscopes. Pap smears and biopsy diagnoses were correlated significantly for MOI ($P < 0.001$), and there were significant improvements ($P = 0.0108$) in accuracy when 3-D, high-definition microscopy was compared with conventional microscopy. The authors found no statistically significant correlation between ASCUS diagnoses that were rendered by using standard microscopes compared with the subsequent biopsy.

**CONCLUSIONS.** Due to enhanced visualization through thick cell clusters, an increased depth of field, light penetration, and resolution, high-definition, 3-D microscopy based on MOI produced superior accuracy compared with conventional light microscopy in evaluating cervical Pap smears. ***Cancer (Cancer Cytopathol)* 2002;96:181–6.** © *2002 American Cancer Society.*

**KEYWORDS: three-dimensional microscopy, Papanicolaou smears, atypical squamous cells of undetermined significance, multiple oblique illumination.**

Since the introduction of the cervical Papanicolaou (Pap) smear, the number of deaths in women due to cervical carcinoma has decreased greatly[1] and has remained relatively constant since the mid-1980s.[2] Although this relatively inexpensive and simple test has affected the mortality due to cervical carcinoma dramatically, it has recently been the subject of numerous articles regarding its sensitivity and specificity as a diagnostic tool. The sensitivity and specificity of the Pap smear reportedly are 80.0% and 99.4%, respectively.[3] A vital


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statistic that has been a prevalent topic within the academic literature, not to mention the media, is the false negative rate of Pap smears that reportedly ranges from 5% to 40%.[1] There are various methods for calculating the false negative rates or error rates in cervical Pap smears.[4–6] Although these numbers are respectable for a screening tool, the common notion is that this test lacks the precision that the general population has come to expect. In addition, it is commonly expected by patients and attorneys that the Pap smear represents a diagnostic tool rather than a screening tool for cervical carcinoma.

The Bethesda System[7] for grading cervical Pap smears is a standardized manner in which to report the diagnoses given to cervical Pap smears. The category *atypical squamous cells of undetermined significance* (ASCUS) is reserved for those smears in which a diagnosis of reactive or inflammatory changes, etc., cannot be distinguished from dysplasia. The ASCUS category was subdivided further into favor reactive, favor dysplasia, etc.[7] These subdivisions have shown that the only useful qualifiers in terms of patient management are those classified as favor reactive or favor high-grade squamous intraepithelial lesion (SIL).[8]

The perceived inadequacies of the cervical Pap smear have been attributed to sampling, screening, or interpretive errors.[5] Unfortunately, no system can completely eradicate an interpretive error by the cytopathologist/technologist. Sampling errors are an inherent pitfall when using cytologic preparations relative to histopathologic examination. Screening errors, in fact, may be reduced by the review of increased numbers of *atypical* cells. However, to our knowledge, no one has addressed the issue that, within any cytologic preparation, there are thick smears, areas, and/or clusters in which the cells in fact are *hidden* and are not evaluated. It is possible that these thick areas harbor diagnostic cells that simply cannot be evaluated. These may be considered screening errors. However, with current microscope technology, these areas cannot be visualized. Thus, this type of error may be an interpretive error.

Multiple oblique illumination (MOI) is a novel lighting technique for transmitted and reflected light microscopes that produces high-definition, three-dimensional (3-D) images directly through the eyepieces. The illumination beams simultaneously generate two tilted views of the specimen, and the observer's eye-brain complex creates the perception of 3-D space (stereopsis). An additional benefit of MOI is that the resulting images have enhanced resolution, depth of field, and contrast.[9] The properties of MOI allow for enhanced visualization through thick cell clusters.[10] We hypothesized that high-definition 3-D microscopy, with its ability to penetrate thick areas, would be useful in evaluating problematic cervical PAP smears, particularly those diagnosed as ASCUS.

## MATERIALS AND METHODS

### Experimental Design

We hypothesized that MOI and 3-D, high-definition microscopy could significantly reduce the false negative rate of cervical PAP smears and increase accuracy. To test this hypothesis, ASCUS PAP smears were evaluated prospectively using both a standard axially illuminated microscope and the Edge R-400 (Edge Scientific, Santa Monica, CA) high-definition, 3-D microscope with MOI. The Pap smears were reviewed in a blinded fashion using both microscopes. The previously reported Pap smear diagnoses were unknown to the reviewer. These newly rendered diagnoses were then compared with the subsequent cervical tissue biopsies, which also were read blinded using conventional microscopy, as the definitive end point. Statistical analyses were then performed.

### Patients and Cervical Pap Smears

Ninety-nine cervical Pap smears and subsequent cervical biopsies were gathered from the Pathology Departments of Robert Wood Johnson Medical School/Cooper Health System (Camden, NJ) and the University of California Davis Medical Center (Sacramento, CA). The smears were selected randomly from patients known to have subsequent tissue biopsies. The smears dated back to 1994. Patient age range from 15 years to 72 years. The original diagnoses were unknown to the reviewer.

### Microscopic Analysis

The Pap smears were blinded and read by a single reviewer (R.R.) using conventional axially illuminated microscopes (Nikon, Melville, NY; or Olympus, Melville, NY) and the R-400 with MOI. Blinded specimens were reviewed first using standard axial illumination microscopy followed by analysis using the R-400 high-definition microscope. The angle of oblique light utilized for the Edge R-400 typically ranged from default (0) to −2. It should be noted that the smears were reviewed en mass, such that diagnoses rendered by the standard microscope could not be known when they were reviewed using the R-400. The slides were examined using standard screening techniques for cytology specimens. Briefly, the slides were scanned using a 10× objective lens in a systematic fashion. Higher power objective lenses were employed when greater magnification was needed. The increased depth of field and light penetration of the

R-400 required additional focusing through multiple planes and depths of thick cell clusters.

### Diagnostic Criteria

After examination of the Pap smears, they were classified using a modification of the Bethesda System.[7] The basic structure of the Bethesda System was kept intact. The ASCUS category was expanded and qualified further using the following scale: favor reactive, inflammatory, repair, etc.; favor + dysplasia; favor ++ dysplasia; and favor +++ dysplasia.

It is important to note that the qualifiers for + *dysplasia* were purely subjective and were not correlated with low-grade, moderate-grade, or high-grade dysplasia; they were simply a measure of how confident the reviewer was that dysplasia was present within the smear. Thus, + *dysplasia* signaled that there was little confidence in the presence of dysplasia, but the atypical cells were not clearly reactive. Conversely, +++ *dysplasia* meant that there was relatively high confidence that there was an SIL present, but not quite enough of the diagnostic criteria were present to render a diagnosis of a low-grade or high-grade SIL. The biopsies were reviewed and diagnosed using the standard criteria for cervical dysplasia.[11] Briefly, biopsies were diagnosed as normal, reactive/inflammatory changes, squamous metaplasia, koilocytosis, or dysplasia (low-grade to high-grade cervical intraepithelial neoplasia [CIN] I–III).

### Scaling/Ranking Diagnoses

For statistical purposes, the rendered diagnoses for Pap smears were scaled and/or ranked as follows: 0) within normal limits; 1) benign cellular changes (reactive, inflammatory, squamous metaplasia, repair); 2) ASCUS, favor reactive (inflammatory, repair, squamous metaplasia); 3) ASCUS, favor + dysplasia; 4) ASCUS, favor ++ dysplasia; 5) ASCUS, favor +++ dysplasia; 6) low-grade SIL (koilocytosis with or without CIN); and 7) high-grade SIL (CIN II–III and carcinoma in situ). The rendered diagnoses for biopsies were ranked as follows: 0) no pathologic diagnosis; 1) reactive, inflammatory, repair, squamous metaplasia; 2) low-grade cervical intraepithelial neoplasia (CIN I) or koilocytosis; 3) moderate-grade to high-grade CIN (II–III)/carcinoma in situ; and 4) invasive carcinoma.

### Statistical Analyses

The true positive (TP) and true negative (TN) rates as well as the false positive (FP) and false negative (FN) rates were calculated for each set, including a Pap smear and its corresponding biopsy specimen, using the following formulas: % TP rate = [TP/(total number of sets reviewed)] $\times$ 100; % FP rate = [FP/(total number of sets reviewed)] $\times$ 100; % FN rate = [FN/(total number of sets reviewed)] $\times$ 100; and % TN rate = [TN/(total number of sets reviewed)] $\times$ 100. We defined the TPs as the number of sets in which the Pap smear diagnosis agreed with the subsequent biopsy. Similarly, the FNs were defined as the number of sets in which the Pap smear failed to show a significant lesion that was found subsequently within the biopsy. FPs were the number of sets in which the smear showed a more significant lesion than was found in the subsequent biopsy. TNs were the number of sets in which both the smear and biopsy diagnoses agreed. These sets essentially were *within normal limits*.

These formulae are slightly different than the currently accepted equation for FN rates (FN rate = [FN/(TP + FN)][4–6]). The formulae that we have described may be better descriptors in this sample of patients with previously diagnosed ASCUS, because, according to the study design, there are no TN diagnoses.

The FP rate, as we have defined it, is also unconventional for the reasons discussed above. We paid particular attention to this statistic for the following reason: The reviewer would be inclined to *over-call* the Pap smear diagnoses using the Edge R-400 microscope. This inclination would be natural, because the study hypothesis was to prove that the R-400 is a better microscope for evaluating Pap smears.

The correlation of Pap smear diagnoses with the subsequent biopsies (axial vs. MOI) were determined independently using the Spearman test of correlation, as provided by SigmaStat (Jandel-SPSS, Chicago, IL). In addition, tests for significant differences between groups using conventional microscopy versus MOI microscopy were performed using the chi-square and McNemar test (also provided by SigmaStat). $P$ values were considered significant if they were $\leq 0.05$. The data generated from both institutions (Robert Wood Johnson/Cooper vs. University of California Davis) were compared directly using a chi-square test (Jandel-SPSS) to confirm the accuracy of MOI at more than one center and to show that conventional microscopy provided similar results from center to center.

## RESULTS

The prospective diagnoses using standard microscopy were compared with the original diagnoses rendered by various pathologists during the course of clinical practice. There was no significant difference between these two groups ($P > 0.05$; McNemar test). Subjectively, it was immediately apparent that the R-400 had better resolution (Fig. 1) compared with the standard microscope (Fig. 2). The 3-D contours of the smear could be seen with great detail. The absolute depth of

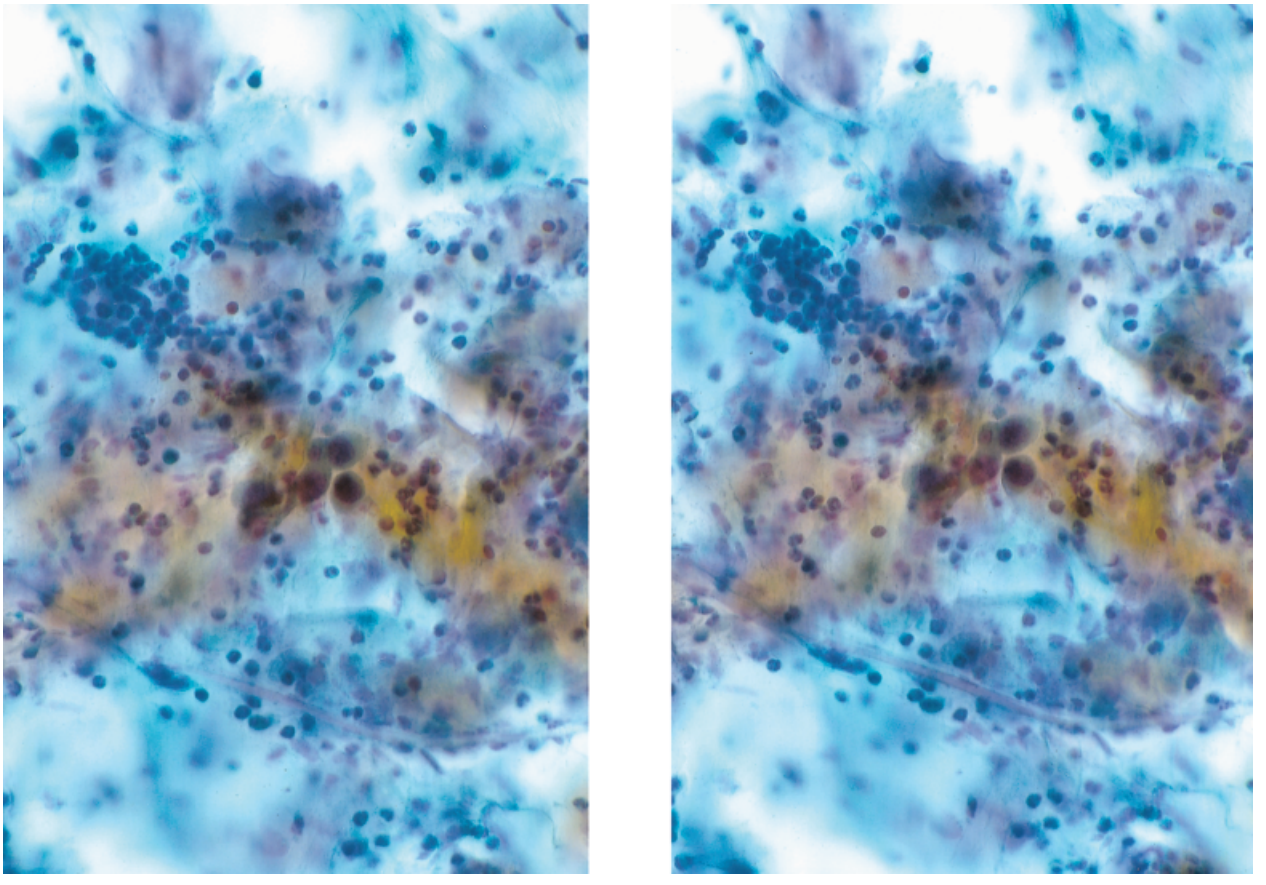

**FIGURE 1.** Stereo, three-dimensional (3-D) image of a cervical Papanicolaou (Pap) smear showing a dysplastic cell within a hyperchromatic, crowded group (Pap stain; original magnification, ×200). Special instructions for 3-D viewing: Stereo pictures should be oriented with the right and left images side by side. The viewer should hold the images at a distance 18–24 inches from the eyes and should cross his or her eyes so that three images are seen; the viewer should focus on the center image to realize the 3-D image (the 3-D image also can be viewed with a specialized lunette available from corresponding author).

field was greater, and the reviewer was left with the impression that the smear had been examined more comprehensively.

The TP, FP, TN, and FN rates for both conventional axial microscopy and 3-D, high-definition MOI microscopy are summarized in Table 1. The scaled Pap smear and biopsy diagnoses were correlated (Spearman rank-order correlation) to determine whether there was a statistically significant association between the Pap smear diagnosis (axial microscopy vs. MOI microscopy) and the subsequent tissue biopsy diagnosis. The correlation coefficient for axial illumination was 0.037, which was not statistically significant ($P = 0.717$). The correlation coefficient for MOI microscopy was 0.36 ($P < 0.001$), which was highly statistically significant.

The diagnoses rendered on the conventional and MOI microscopes were compared using the McNemar test to determine the effectiveness of the 3-D MOI microscope in detecting significant changes in the Pap smears (Table 2). The Pap smears were determined to be correct or incorrect by direct comparison with the biopsy. There were statistically significant differences ($P = 0.0108$) between the diagnoses rendered using MOI microscopy compared with conventional microscopy. The Robert Wood Johnson/Cooper data did not reflect any statistically significant difference (axial microscopy, $P = 0.53$; MOI microscopy, $P = 0.74$; chi-square test) compared with the data from the University of California Davis.

## DISCUSSION

ASCUS remains the bane of the cytology arena. Despite the attempts of the Bethesda System to standardize this category, suboptimal diagnoses continue to be rendered at an annual cost of $6 billion.[12] Using our scale for statistical purposes, we found no statistical correlation between diagnoses rendered using a standard microscope versus the subsequent biopsy. However, there was a highly statistically significant corre-

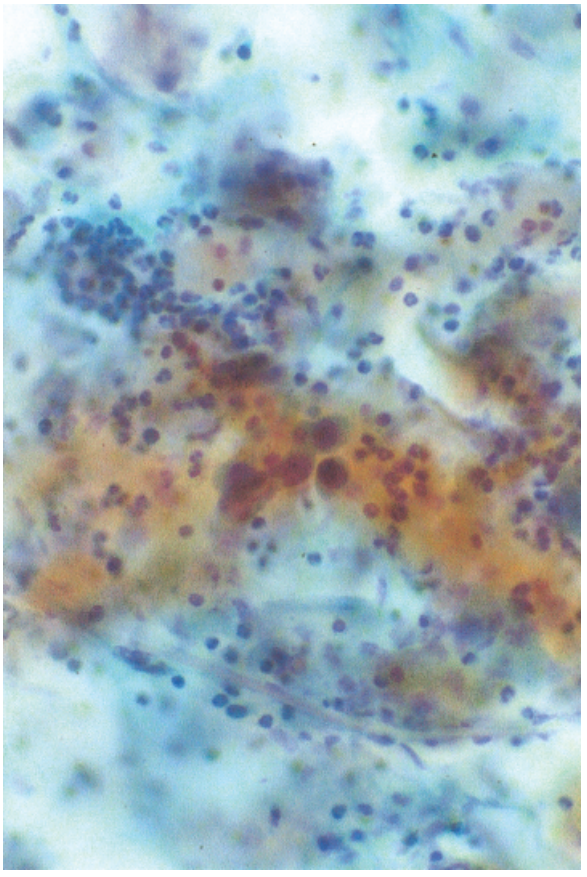

**FIGURE 2.** Conventional two-dimensional image of the same field shown in Figure 1 (Papanicolaou stain; original magnification, ×200).

**TABLE 1**
**Papanicolaou Smear True and False Positive and Negative Results Obtained Using Conventional versus High-Definition Three-Dimensional Microscopy with Multiple Oblique Illumination**

| Pap smear screening results[a] | Conventional microscopy (%) | MOI microscopy (%) |
|---|---|---|
| True positive | 60 (60.6) | 73 (73.7) |
| False positive | 16 (16.2) | 14 (14.1) |
| True negative | 0 (0.0) | 0 (0.0) |
| False negative | 23 (23.2) | 12 (12.1) |

Pap: Papanicolaou; MOI: multiple oblique illumination.
[a] Number of patients (%).

lation ($P < 0.001$) between high-definition, 3-D microscopy based on MOI diagnoses and the subsequent biopsy. The absence of a statistical correlation when comparing axial microscopy with subsequent biopsy results is somewhat alarming, although it is important to note that this represents a statistical test that is not typically performed when quality control/assurance issues are examined regarding ASCUS Pap

**TABLE 2**
**McNemar Contingency Table: Papanicolaou Smear Positive and Negative Results Obtained Using Conventional versus High-Definition Three-Dimensional Microscopy with Multiple Oblique Illumination (All Patients)**

| | 3-D MOI microscopy | |
|---|---|---|
| Conventional microscopy[a] | Positive | Negative |
| Positive | 56 | 6 |
| Negative | 20 | 17 |

3-D: three-dimensional; MOI: multiple oblique illumination.
[a] Positive, concordant; negative, discordant ($P = 0.0108$).

smears and subsequent biopsies. In addition, when the MOI and standard microscope diagnoses were compared using the McNemar test, there was a highly statistically significant difference ($P = 0.0108$) between the two sets of diagnoses.

Several important points follow from these statistical analyses. Our FN percentage (FNP; 12.1%), as we calculated it (FNP = [FN/total no. of sets]) was lower using MOI microscopy compared with axial microscopy (23.2%). This formula, as stated previously, is slightly different than the currently accepted equation for FN rates (FN rate = [FN/(TP + FN)][4–6]). The former equation (FNP), in all likelihood, is a better descriptor in this sample of patients with previously diagnosed ASCUS, because there are no TN diagnoses according to the definitions used in this study. The FP percentages were similar and were actually slightly lower using the MOI microscope versus the standard microscope (16.2% vs. 14.1%, respectively). Thus, there was no evidence of *over-calling* Pap smear diagnoses using MOI microscopy. The TP percentage was increased using MOI microscopy (73.7%) compared with conventional microscopy (60.6%). Thus, the accuracy of the Pap smear improved statistically significantly ($P = 0.0108$; McNemar test) using MOI microscopy, as shown by the increased TP rates and decreased FP rates.

We have not reported the sensitivities or specificities of this technology for several reasons. First, we preselected the diagnostic category of ASCUS with the expectation that there would be no *normal* smears. Thus, without a TN, the sensitivities and specificities are not appropriate statistics. To truly obtain these numbers, a large prospective study screening all diagnostic categories of cervical Pap smears using MOI microscopy would be necessary.

There are several possible reasons why MOI produces better results. MOI provides an increased depth of field and greater contrast for microscopic examina-

tion. In addition, the light penetration through thick cell clusters is much greater.[10] However, the most important feature of the MOI system is the ability to provide 3-D imaging directly through the microscope eyepieces. Stereopsis is an example of hyperacuity, i.e., acuity that is finer than the separation between the cones in the retina. It is a powerful cue for figure/background discrimination, which helps the figure *pop out* from the background; the scene is viewed as a whole, all at once. Stereo is a cue that makes things *pop out* easily, so the observer does not have to search in a serial, time-consuming fashion.[13]

Errors in cytology, as discussed above, can be attributed to sampling, screening, or interpretive errors.[5] We hypothesized that thick areas or cell clusters that could be visualized due to the inadequacies of standard light microscopy, in fact, would be visible using MOI microscopy. These thick areas have recently been deemed hyperchromatic crowded groups (HCGs) by Demay.[4] It has also been proposed that these HCGs represent interpretive errors that have not been emphasized sufficiently in the literature. These HCGs may harbor serious lesions.[5] The errors lead to FN readings and are 24 times more likely to occur in samples that contain less than 50 abnormal cells.[14–17] Thus, any technology that enhances the evaluation of these HCGs, in theory, should decrease FN Pap smear results, as we have shown.

With regard to the FP rate, we used the subsequent tissue biopsy as the *definitive* diagnosis. The true diagnosis, however, depends on long-term patient outcome, i.e., the development of dysplasia, carcinoma, or a benign change. In practical terms, this end point is unattainable. Thus, the subsequent biopsy diagnosis, as we have used it herein, may be superior to rescreening by a committee of *experts* that typically is done in quality-control or review studies of Pap smear accuracy. Indeed, it has been shown that there is very little consensus on rereviews of ASCUS Pap smears.[5] Thus, although our FP percentage, as we have defined it, is roughly 14%, it may take into account sampling error during biopsy and subsequently may not be a true representation of the FP rate. Long-term patient follow-up will be necessary to make this differentiation.

The interim management of patients with abnormal cervical cytology has been problematic and is a strain on the health care system.[12] Repeat cervical Pap smears and/or cervical biopsies are the options for management but are not without associated costs and risks. We have shown that 3-D microscopy employing MOI can improve the accuracy of the cervical Pap smear. In addition, 3-D MOI microscopy is as simple to use as a conventional microscope and potentially may replace these microscopes in the cytology laboratory. In summary, we feel that this new technology is an invaluable tool and may provide more definitive diagnoses, resulting in enhanced patient care and cost savings.

## REFERENCES


1. Koss LG. The Papanicolaou test for cervical cancer detection: a triumph and tragedy. *JAMA.* 1989;34:737–743.
2. Kosary CL, Ries LAG, Miller BA, Hankey BF, Harras A, Edwards BK, editors. SEER cancer statistics review, 1973–1992: tables and graphs. National Institutes of Health publication no. 96-2789. Bethesda: National Cancer Institute, 1995.
3. Soost HJ, Lange HJ, Lehmacher W, Rutting-Kullman B. The validation of cervical cytology: sensitivity, specificity, and predictive values. *Acta Cytol.* 1991;35:8–14.
4. Demay RM. Common problems in Papanicolaou smear interpretation. *Arch Pathol Lab Med.* 1997;121:229–238.
5. Davey DD. Quality and liability issues with the Papanicolaou smear: the problem of definition of errors and false-negative smears. *Arch Pathol Lab Med.* 1997;121:267–269.
6. Naryshkin S. The false negative fraction for Papanicolaou smear: how often are abnormal smears not detected by a standard screening cytologist? *Arch Pathol Lab Med.* 1997; 121:270–272.
7. Kurman RJ, Solomon D. The Bethesda System for reporting cervical/vaginal cytologic diagnoses: definitions, criteria, and explanatory notes for terminology and specimen adequacy. New York: Springer-Verlag, 1994:30–43.
8. Genest DR, Barbara D, Lee KR, Sheets E, Crum CP, Cibas ES. Qualifying the cytologic diagnosis of "atypical squamous cells of undetermined significance" affects the predictive value of a squamous intraepithelial lesion on subsequent biopsy. *Arch Pathol Lab Med.* 1998;122:338–341.
9. Greenberg G, Boyde A. Convenient and controllable direct view 3D imaging in conventional light microscopes: approaches via illumination and inspection. *Proc R Microsc Soc.* 1997;32:97–101.
10. Hewitt C, Tarnoff M, Goodman M, Ramsamooj R, Chin V, Doolin EJ. Use of three dimensional microscopy with multiple oblique illumination in evaluating cytopathology preparations. *Anal Quant Cytol Histol.* 1999;21(2):139–142.
11. Crum CP, Nuovo GJ. The cervix. In: Sternberg SS, editor. Diagnostic surgical pathology. 3rd ed. New York: Raven Press, 1994:2055–2090.
12. Kurman RJ, Henson DE, Herbst AL, Noller KL Schiffman MH. Interim guidelines for management of abnormal cervical cytology. *JAMA.* 1994;271:1866–1869.
13. Nakayama K, Silverman GH. Serial and parallel processing of visual feature conjunctions. *Nature.* 1986;320:264–265.
14. DiBonito L, Falconiert G, Tomasic G, Colnutti I, Bonifacio D, Didine S. Cervical cytopathology: an evaluation of its accuracy based on cytohistologic comparison. *Cancer.* 1993;15: 3002–3006.
15. Koss LG. Cervical Pap smear: new directions. *Cancer.* 1993; 71(Suppl 4):1406–1412.
16. Mitchell H, Medley G. Differences between Papanicolaou smear with correct and incorrect diagnosis. *Cytopathology.* 1995;6:368–375.
17. Pearson DI, Dawood MY, Hammond CB. Medical legal issues in gynecology. Green's gynecology: essentials of clinical practice. 4th ed. New York, 1990:24–26.