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Optical Imaging of the Cervix

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Recent advances in fiber optics, sources and detectors, imaging, and computer-controlled instrumentation have stimulated a period of unprecedented growth in the development of photonics technologies for a wide variety of diagnostic and therapeutic clinical applications. These include the application of quantitative optical spectroscopy and imaging for the detection of precancerous lesions in the uterine cervix, a topic of interest at the Second International Conference on Cervical Cancer, which was held April 11–14, 2002. Investigators have applied the Littenberg method of emerging technology assessment to new optical methods used to detect cervical neoplasia. Currently, such technologies as fluorescence spectroscopy (the combination of fluorescence and diffuse reflectance spectroscopy), tri-modal spectroscopy, and light-scattering spectroscopy that probe the spectral characteristics of tissue are being investigated. Optical technologies that create images of subcellular structure without biopsy subsequent to pathology that currently are under investigation include in vivo confocal imaging and optical coherence tomography. Numerous small studies have demonstrated the potential of these optical technologies. What remains to be elucidated are the fundamental biophysical origins of variations in remitted optical signals between normal and dysplastic tissue. Large multicenter randomized controlled trials are needed to confirm the detection and imaging capabilities of optical technology. Furthermore, the development of contrast agents that could boost detection with these technologies is needed, and basic biologic characterization of signals should be pursued. Applying the Littenberg assessment will help ensure that superior, not simply alternative, technologies are implemented. ***Cancer*** **2003;98(9 Suppl):2015–27.**
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Recent advances in fiber optics, sources and detectors, imaging, and computer-controlled instrumentation have stimulated unprecedented growth in the development of photonics technologies for a wide variety of diagnostic and therapeutic clinical applications. The use of noninvasive optical techniques for the early detection of precancerous conditions is one rapidly emerging area within the field of biophotonics. Quantitative optical spectroscopy and imaging can improve the current clinical strategies for the screening and diagnosis of epithelial precancerous lesions in a variety of organ sites, including the uterine cervix. Detection of cervical precancerous lesions is a particularly important clinical application of emerging optical technologies because of the potential for improvement in the current standard of care both in the U.S. and abroad. Greater than $6 billion is spent annually in the U.S. in managing low-grade cervical lesions, and significant monetary resources are consumed in monitoring and treating lesions unlikely to progress to malignancy (findings such as abnormal squamous cells of unknown significance and low-grade

squamous intraepithelial lesions [LGSILs]). By eliminating unnecessary biopsies and treatments, optical technologies could have a significant impact on care in the U.S. through cost reduction. In developing countries, cervical cancer often goes undetected because of insufficient personnel and resources to perform adequate screening and diagnosis. Optical technologies could greatly improve care worldwide by providing automated, machine-read diagnosis at a screening visit. Automation would permit use of the technology by health care workers with less clinical training than that of physicians and nurse practitioners.

Both industrial and academic research groups believe that the screening and detection of cervical cancer precursors could be improved significantly by optical technologies that automate and decrease the cost of screening and detection while improving accuracy. In the current study, we summarize the current research in developing emerging optical technologies for the detection of cervical precancerous lesions based on such spectroscopic approaches as fluorescence spectroscopy, diffuse reflectance spectroscopy, or trimodal spectroscopy and on direct high-resolution imaging methods, including confocal reflectance microscopy and optical coherence tomography. After a review of current research using each of these optical modalities, we discuss potential future research directions for the optical assessment of cervical neoplasia.

OPPORTUNITIES FOR EMERGING TECHNOLOGIES

Although the introduction of organized screening and detection programs has decreased cervical cancer mortality and morbidity, significant gaps in care persist. Because the Papanicolaou (Pap) smear has a reported average sensitivity of $\leq$ 58% and a specificity of 69%,[1–3] many lesions are missed or overcalled. To decrease the impact of false-negative cytology findings, the test must be repeated annually, making screening by cytology cost-ineffective. Because of false-positive Pap test results, many women are unnecessarily referred for colposcopy, resulting in both considerable anxiety and economic cost.

Expert colposcopy has an average sensitivity of 96% (average colposcopy has a sensitivity reportedly estimated as low as 79% by some) and a specificity of 48%.[4] Small, 1-quadrant lesions are missed in 30% of cases,[5] and in 1 study, 58% of microinvasive tumors were not detected by expert colposcopy.[6] Thus, multiple biopsies are required to confirm diagnosis. This low sensitivity allows for the detection of most cancers, but the specificity suggests that many lesions are overcalled, leading to many unnecessary biopsies. Those patients with accurately identified high-grade lesions may wait 2 weeks for confirmation and may be lost to follow-up. Until recently, considerable controversy existed regarding the evaluation and treatment of low-grade disease because of the difficulty of determining which lesions will progress to cancer or regress to normal. Consensus guidelines were promulgated in 2002 in an effort to resolve the issue.[7]

Emerging technologies have the potential to address these gaps. Optical spectroscopy and imaging provides a tool with which to examine the entire epithelial volume in situ and provides an objective assessment of the biochemical and morphologic status. Used for screening and diagnosis, these technologies have the potential to provide immediate and accurate diagnostic information, potentially reducing both the costs associated with unnecessary biopsies and treatment delays.

Although these emerging techniques appear promising, all emerging technologies should be evaluated in a systematic way that allows them to be optimized during development. Technology assessment provides an explicit methodology with which to achieve this goal. In 1992, Littenberg proposed five levels of technology assessment: biologic plausibility, technical feasibility, intermediate effects, patient outcomes, and societal outcomes.[8] Biologic plausibility refers to whether current understanding of the biology and pathology of the disease in question can support the technology. Technical feasibility refers to the level of assessment in which physicians can safely and reliably deliver the technology to the intended patients. Intermediate effects assess the sensitivity and specificity in a relevant population. Patient outcomes assess whether the technology improves the patients' health and societal outcomes assess the cost and ethical implications of a technology. Wortman and Saxe described the Fineberg proposal for a hierarchy of evaluation of diagnostic technologies: technical capacity (whether the device performs reliably and delivers accurate information), diagnostic accuracy (whether the test result improves it), diagnostic impact (whether the test result influences the pattern of testing or subsequent testing or replaces other tests) therapeutic impact (whether the test result influences the selection and delivery of therapy), and patient outcome (whether test performance contributes to the improved health of the patient).[9] The Littenberg classification can be applied to emerging or existing technologies, whereas that described by Wortman and Saxe is best suited to existing technologies.[8,9]

The next section focuses on the emerging technology of fluorescence spectroscopy. Following the Littenberg model of technology assessment, we examine the biologic basis of the technology first.

BIOLOGIC BASIS OF CERVICAL TISSUE FLUORESCENCE

A number of pilot trials have been performed that demonstrate that fluorescence spectra can be measured and analyzed safely to classify tissue as normal or diseased.[10–12] However, the underlying biochemical and morphologic changes that occur in disease and cause such spectral differences to our knowledge are only beginning to be elucidated. This section reviews research into the biologic basis for changes in optical spectra as cervical precancerous lesions develop.

Fluorophores Found in Cervical Tissue

A number of naturally occurring fluorophores are present in cervical tissue. The pyridine nucleotides and the flavins play an important role in cellular energy metabolism.[13] Nicotinamide adenine dinucleotide is the major electron acceptor. Its reduced form is NADH, and the reduced nicotinamide ring is fluorescent.[14] Flavin adenine dinucleotide is the other major electron acceptor; the oxidized form (FAD) is fluorescent, whereas the reduced form ($FADH_2$), is not.[15] Several investigators have shown that the endogenous fluorescence of nearly 500 nanometers (nm) when excited in the near ultraviolet region is weaker in tumor tissue compared with normal surrounding tissues.[16,17] Differences may lie in the decrease in the oxidized forms of flavins and the relative amount of NADH in malignant tissues.

The aromatic amino acids tryptophan, tyrosine, and phenylalanine contribute to protein fluorescence.[14] Tryptophan typically accounts for the majority of protein fluorescence, and its emission is sensitive to environmental polarity. At excitation wavelengths $>$ 295 nm, only tryptophan is excited. When the excitation wavelengths are between 280 and 295 nm, both tyrosine and tryptophan are excited; however, energy transfer from tyrosine to tryptophan is quite common, and the emission may be dominated by that of tryptophan. When the excitation wavelengths are $<$ 280 nm, all 3 aromatic amino acids can be excited, but the quantum yield of phenylalanine is relatively low compared with that of tryptophan and tyrosine.

Autofluorescence has been noted in the structural proteins collagen[18] and elastin.[19] Collagen fluorescence is associated with cross-links,[18] and elastin autofluorescence also is believed to be associated with cross-links, which are autofluorescent with an excitation maximum at 325 nm and an emission maximum at 400 nm.[18,20]

AGE DEPENDENCE OF CERVICAL FLUOROPHORES

Dr. Richards-Kortum's group has developed tissue culture techniques to view the fluorescence of transverse slices of living cervical tissue directly. Her group has used this tissue-culture model to examine how cervical fluorescence varies in different groups of patients. These studies indicate that there are important changes that occur both with patient age[21] and the presence of dysplasia.[22]

A microtome that can rapidly prepare fresh tissue slices has been described by the Richards-Kortum group in the work by Brookner et al.[21] This device can produce fresh tissue slices of a consistent thickness with minimal trauma. To examine the effects of patient age on the pattern of autofluorescence, the researchers prepared fresh tissue slices of the cervix using this microtome.[21] Cervical biopsy specimens (2 mm $\times$ 4 mm $\times$ 1 mm) were obtained from women seen at The University of Texas M. D. Anderson Cancer Center Colposcopy Clinic and the Lyndon B. Johnson Hospital Colposcopy Clinic in Houston. (Informed written consent was obtained from all participating women.) Biopsy specimens were immediately placed in chilled culture medium and then embedded in agarose. The Krumdieck Tissue Slicer (Alabama Research and Development, Munford, AL) was used to obtain 200-μm transverse fresh tissue slices, which were cut in perpendicular fashion rather than parallel to the epithelial surface so that each slice could be used to image fluorescence intensity as a function of depth. Fluorescence images were obtained from tissue slices within 1.5–5 hours of biopsy being performed.

A Zeiss Axiophot 410 inverted fluorescence microscope (Zeiss, Inc., Thornwood, NY) was used to examine the unstained tissue slices. Fluorescence images at 380-nm and 460-nm excitation were collected using a $\times$10 objective and a 5-second exposure time. A filter cube with filter sets I (BP 380 exciter filter, FT470 dichroic, and 420 long pass filter) and II (BP455 exciter filter, FT470 dichroic mirror, and GG495 filter) was used in the studies described. All the filters were obtained from Omega Optical (Brattleboro, VT). The appropriate dark current image was subtracted from each fluorescence image. After fluorescence microscopy, 4-μm sections were made for histologic evaluation and were read by a board-certified pathologist to provide a diagnosis.

Researchers collected brightfield and fluorescence images, with recognizable epithelium and stroma, from 31 colposcopically normal cervical biopsy samples from 21 patients.[21] Hematoxylin and eosin stained sections from the 31 colposcopically normal biopsy specimens were diagnosed as normal. Based

on the fluorescence pattern, images from the normal cervix were divided into 3 categories (Fig. 1): 1) slices with bright epithelial fluorescence, which generally was brighter than the stromal fluorescence, 2) slices with similar fluorescence intensities in the epithelium and stroma, and 3) slices with weak epithelial fluorescence and strong stromal fluorescence. A strong correlation was observed between age and the fluorescence pattern of the normal biopsies. The average age of the patients in Group 1 was 30.9 years, and only 1 of the 12 patients was postmenopausal; the average age of the women in Group 2 was 38.0 years, and 3 of the 7 patients were postmenopausal; in Group 3, the average age was 49.2 years, and 3 of the 4 patients were postmenopausal. The average age of the patients in each group demonstrated a dramatic correlation between fluorescence pattern and patient age. Histologic findings suggested an association with collagen. An increase in collagen fluorescence with age is biologically plausible because collagen fluorescence is attributed to its cross-links[20] and the amount of cross-linking increases with age.[23] These results suggest a biologic basis for the increased fluorescence intensity observed when fluorescence is measured in vivo from the intact cervix in older, postmenopausal women.[16]

To assess how fluorescence patterns change with the presence of cervical neoplasia, Richards-Kortum's group performed a similar study to characterize autofluorescence. Thirty-four patients were enrolled in the study who ranged in age from 21–61 years (mean patient age, 33.4 $\pm$ 12.5 years). For 20 patients, both colposcopically normal and colposcopically abnormal biopsy specimens were obtained, sliced, and imaged successfully. In an additional six patients, either the colposcopically normal or abnormal biopsy specimen was sliced and imaged. In 15 patients with both colposcopically normal and abnormal specimens, hematoxylin and eosin stains of both were made. In an additional 3 of the 20 patients with paired specimens, only stained specimens from the colposcopically normal biopsy were found to be of acceptable quality. In this study, tissue was considered normal when classified pathologically as normal, as normal tissue characterized by inflammation, or as normal tissue with focal clusters suggestive of koilocytosis without SILs. Tissue classified as abnormal included tissue with LGSILs or high-grade squamous intraepithelial lesions (HGSILs). For the 12 patients with histologically confirmed normal and abnormal tissue slices, 8 patients had LGSILs and 4 patients had HGSILs. A total of 90 individual slices were imaged and subsequently examined by a pathologist. Of these, 55 slices were normal, 30 slices were abnormal (17 with LGSILs and 13 with HGSILs), and 5 could not be evaluated.

When comparing the fluorescence patterns of the normal and precancerous cervix, researchers found that fluorescence intensity increased in the epithelium of SILs but not in the normal tissue, whereas the fluorescence in the stroma significantly decreased (Fig. 2). The ratio of mean epithelial fluorescence to mean stromal fluorescence was computed for all paired samples at 380-nm excitation. The average ratios were found to be 0.55 $\pm$ 0.21 and 1.06 $\pm$ 0.23 for the normal and abnormal samples, respectively. Using a Student *t* test for paired data, the researchers found these ratios to be statistically significantly different ($P < 0.0002$). The average epithelial fluorescence was increased in the samples with SILs (109.3 $\pm$ 41.4) compared with the normal samples (85.8 $\pm$ 32.4). The means were found to be statistically significantly different using a two-tailed Student *t* test for paired data ($P < 0.036$). In contrast, the average stromal fluorescence was markedly reduced in the dysplastic samples (104.2 $\pm$ 37.2) compared with the normal samples (160.7 $\pm$ 42.6). Between the normal and abnormal groups, the intensities of fluorescence in the stroma were statistically significantly different ($P < 0.001$ using a two-tailed Student *t* test for paired data).

At 460-nm excitation, the average epithelial fluorescence was slightly decreased in the dysplastic samples (98 $\pm$ 43) compared with the normal samples (110 $\pm$ 61) but not in a statistically significant manner. The average stromal fluorescence was reduced in the dysplastic samples (93 $\pm$ 35) compared with the normal samples (137 $\pm$ 49). Between the normal and abnormal groups, the intensities of fluorescence in the stroma were statistically significantly different ($P < 0.005$ using a two-tailed Student *t* test for paired data).

The studies discussed in this section demonstrate that fresh tissue sections can provide a valuable model system for investigating the autofluorescence patterns of normal and abnormal cervical tissue. Significant epithelial fluorescence was observed in the majority of the short-term tissue cultures, and the fluorescence intensity increased at 380-nm excitation in dysplastic samples compared with normal samples from the same patient. NADH is likely the source of this epithelial fluorescence. Supporting this hypothesis, fluorescence measurements of ectocervical cells from primary cultures and 2 cervical cancer cell lines demonstrated fluorescence consistent with NADH at wavelengths ranging 340–380-nm excitation. Assuming that NADH is the dominant epithelial fluorophore at 380-nm excitation, the increased NADH fluorescence found in SILs suggests a higher metabolic rate in the abnormal areas. This would create an increased

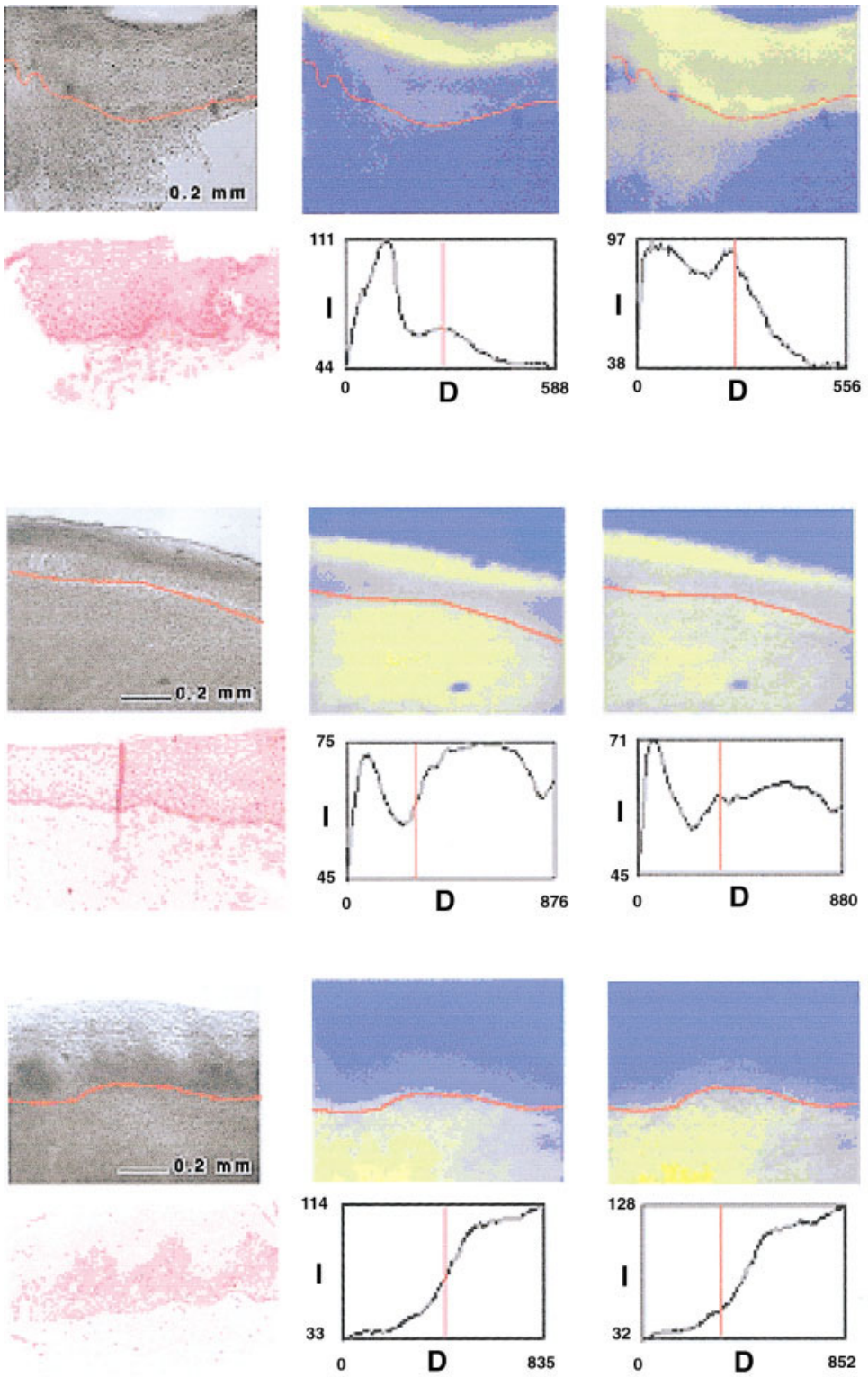

FIGURE 1. Three patients were represented by six images each, and each patient was representative of each of the age groups. (Top)In the first set of 6 images (as in each of the 2 remaining sets), in the top row, the brightfield image (left) is followed by a fluorescence image at 380-nanometer (nm) excitation (middle) and another at 460-nm excitation (right). In the second row, hematoxylin and eosin-stained tissue (left) is followed by two graphs. In the graphs, fluorescence intensity is plotted as a function of depth for fluorescence at 380-nm excitation (middle) and at 460-nm excitation (right). This first set of 6 images is from a 20-year-old woman (Group 1) whose pathology report noted chronic inflammation but reported the tissues to be otherwise normal. The second set of 6 images is from a 40-year-old woman (Group 2) with normal pathology and the third set of 6 images is from a 49-year-old postmenopausal woman (Group 3) whose pathology, although normal, was characterized by a focal area suggestive of koilocytosis.

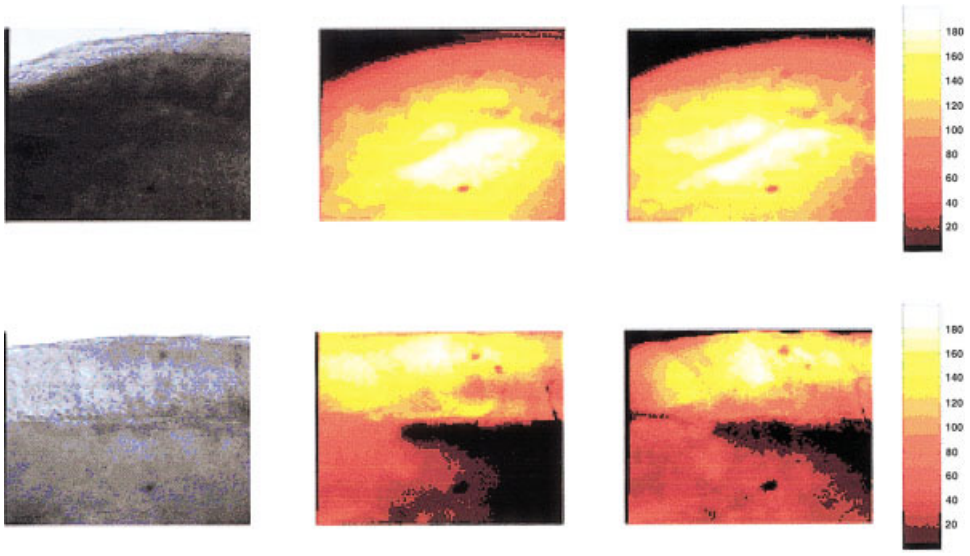

FIGURE 2. Images are from short-term tissue cultures from normal (top row) and neoplastic (bottom row) cervical tissue. Left to right: illustrations are Brightfield images (left) and fluorescence micrographs at 380-nanometer (nm) excitation (middle) and 460-nm excitation (right). As neoplasia develops, the nicotinamide adenine dinucleotide (NADH) fluorescence of the epithelium increases, and the collagen fluorescence of the stroma decreases.

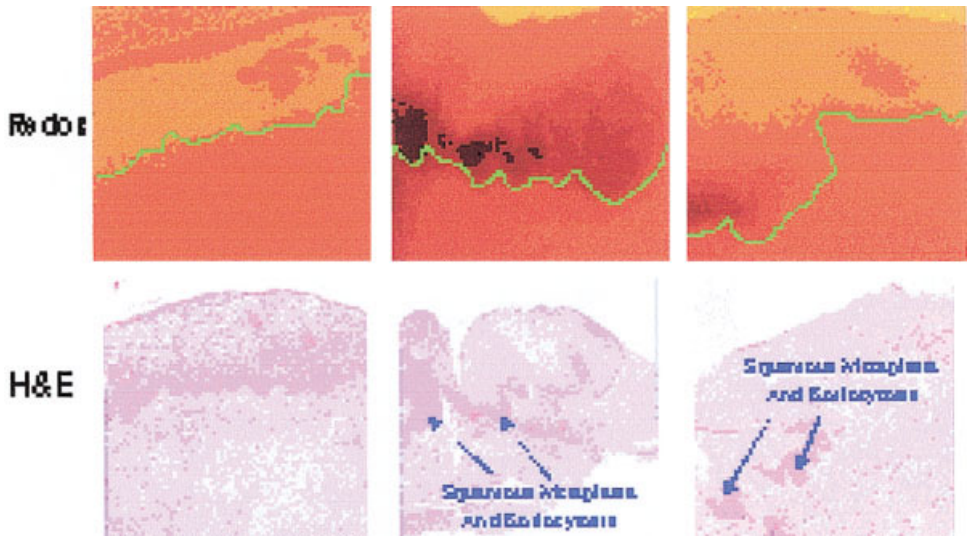

FIGURE 3. False color images (top row) indicate the redox ratio calculated from fluorescence images of short-term tissue cultures of normal (left) and neoplastic (middle and right) cervical tissue. High redox ratios were indicated in orange, whereas low values were in black. The green line indicates the basement membrane. Hemotoxylin and eosin-stained sections of the same samples also are shown (bottom row). Note that areas of reduced redox ratio correspond directly to areas of neoplasia in the hematoxylin and eosin-stained sections.

concentration in the reduced electron carrier NADH and a decreased concentration in the oxidized electron carrier FAD. To determine whether the fluorescence images might contain evidence of metabolic changes between normal and precancerous tissue, we calculated redox images by dividing the 460-nm excitation image by the sum of the 380-nm and 460-nm excitation images (Fig. 3). Marked changes in redox in the precancerous tissue were observed in approximately 33% of the samples. In these cases, redox ratios in the regions of dysplasia (range, 0.1–0.2) were 17–40% of the average epithelial redox ratio of the normal tissue (range, 0.5–0.6).

These results indicate that there is biologic plausibility to support the application of fluorescence methods to discriminate between normal and abnormal cervical tissue. The primary observed difference between normal and dysplastic tissue was a significant

decrease in the stromal fluorescence signal of dysplastic tissue compared with paired normal tissue. The next steps in the Littenberg method of emerging technology assessment are to evaluate technical feasibility and intermediate outcomes. To our knowledge, a number of clinical trials have been conducted to date to evaluate whether cervical fluorescence spectra can be measured in vivo and to assess the sensitivity and specificity associated with fluorescence-based diagnosis relative to a gold standard. The engineering approaches and outcomes are reviewed in the next section.

Clinical Measurements and Analysis of Cervical Tissue Fluorescence

Measurement of in vivo tissue fluorescence from the cervix with good signal-to-noise ratios has been demonstrated in a number of clinical trials. A variety of instrumentation approaches have been pursued. Initially, approaches that concentrated on measuring fluorescence emission spectra from a limited number of cervical sites were introduced. This has been followed rapidly by imaging approaches in which images of the entire cervix are obtained at a reduced number of excitation and emission wavelength combinations. Because cervical tissue contains a number of chromophores with varying excitation spectra, the choice of excitation wavelength(s) can have a dramatic impact on the diagnostic performance.

Instrumentation and Measurement: Point Spectroscopy

Fluorescence spectra can be measured in three primary ways, and when developing fluorescence-based algorithms for the detection of cervical precancerous lesions, the first step is to decide which approach provides the most diagnostically useful results. A fluorescence emission spectrum is produced when the excitation wavelength is fixed and the emission wavelength is scanned. An excitation spectrum is produced when the emission wavelength is fixed and the excitation wavelength is scanned. An excitation emission matrix (EEM) contains fluorescence intensities as a function of excitation and emission wavelength; it is determined by measuring a series of excitation or emission spectra. The fluorescence EEM provides a complete characterization of the fluorescence properties of a sample; individual fluorescence excitation or emission spectra contain a subset of this information because not all chromophores absorb and emit at all wavelengths. In the 1990s, when in vivo cervical fluorescence measurements initially were reported, optical instrumentation was not available to measure fluorescence EEMs in vivo. Thus, initial research focused

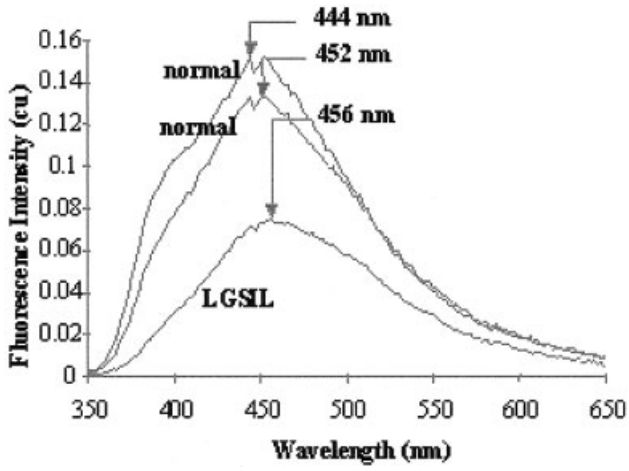

FIGURE 4. Typical spectra measure in the normal cervical tissue and dysplastic cervical tissue in one patient; grade squamous intraepithelial lesion. c.u.: corrected units.

on determining which excitation wavelengths yielded the most diagnostically useful emission spectra.

Richards-Kortum et al. measured fluorescence EEMs in vitro from 18 pairs of normal and abnormal biopsy specimens collected at colposcopy from 18 women.[24] This study demonstrated that the greatest differences in the emission spectra of nondiseased and diseased cervical tissues in vitro occur at excitation wavelengths near 340-nm, 380-nm, and 460-nm excitation, with maximal differences at 340-nm excitation. Based on this work, we developed instrumentation to measure fluorescence emission spectra in vivo at a single excitation wavelength. Using this system, we measured spectra at 337-nm excitation from 114 cervical sites in vivo from a group of 28 patients.[16,25] Figure 4 shows typical spectra at 337-nm excitation from a single patient. As tissue becomes potentially precancerous, the fluorescence intensity drops and the peak emission wavelength shifts to the red. A two-stage spectroscopic algorithm based on empirically determined spectral parameters could differentiate between 1) SILs and normal epithelia and 2) HGSILs and LGSILs. Both stages of the algorithm performed with a similar sensitivity and significantly improved specificity compared with those of colposcopy in expert hands.[25] However, at this excitation wavelength, spectra of columnar normal epithelium are indistinguishable from that of SILs. Furthermore, because the fluorescence intensities varied significantly from patient to patient, the algorithm required measurement from a site known to be normal. Spectra from all tissue sites to be classified by the algorithm were divided by the intensity of the spectrum from this known normal site.

To explore whether additional excitation wave-

lengths could provide better diagnostic ability, researchers modified the instrumentation so that fluorescence emission spectra could be measured at three excitation wavelengths in vivo. The choice of excitation wavelength was based on in vitro EEM measurements and 337 nm, 380 nm, and 460 nm were selected. Tissue spectra from 40 patients at 337-nm and 380-nm excitation and 24 patients at 337-nm and 460-nm excitation demonstrated that the diagnostic potential of spectra at these excitation wavelengths complement that at 337-nm excitation[26] and indicate that tissue spectra at all 3 excitation wavelengths are necessary for the development of an optimal spectroscopic algorithm for the differential diagnosis of SILs in vivo. Richards-Kortum et al. conceived a method to develop diagnostic algorithms that overcame limitations of the empirically derived two-stage algorithm.[27] Preprocessing methods that do not require a priori information were used to calibrate for interpatient and intrapatient variation in tissue fluorescence spectra. Principal component analysis (PCA) was used to dimensionally reduce each type of preprocessed spectral data with minimal information loss. Finally, a probability-based classification algorithm was developed using logistic discrimination. This Bayesian algorithm classifies cervical tissue using three rules: the first classifies samples as squamous normal (SN) or not based on normalized fluorescence spectra; the second classifies the remaining samples as columnar normals (CN) or not based on normalized, mean-scaled fluorescence spectra; and the third classifies the remaining samples as LGSILs or HGSILs based on normalized fluorescence spectra.

Tissue spectra were acquired at all 3 excitation wavelengths from 100 patients in vivo. Tissue spectra at multiple excitation wavelengths were analyzed to determine whether the diagnostic performance of each constituent algorithm developed previously could be improved using tissue spectra at a combination of two or three excitation wavelengths rather than at a single excitation wavelength. In summary, fluorescence spectra at multiple excitation wavelengths can be used to develop composite diagnostic algorithms for the differential diagnosis of 1) SILs and 2) HGSILs with a similar sensitivity and significantly improved specificity compared with colposcopy in expert hands. The original algorithm was based on analysis of the complete emission spectra at the 3 excitation wavelengths, which were comprised of 161 intensities at different excitation emission wavelength pairs. Using component loadings calculated from PCA, the researchers identified a subset of 15 fluorescence intensities at different excitation emission wavelength pairs. An algorithm based on this reduced dataset performed with a minimal decrease in predictive ability compared with the use of the entire emission spectra,[27] significantly reducing the cost of the instrumentation required for clinical implementation. Table 1 summarizes the performance of this algorithm in relation to the standards of care.

TABLE 1
Performance of Fluorescence and Standards of Care for the Detection of Cervical Squamous Intraepithelial Lesions

Technique	Sensitivity	Specificity
Pap smear	62% ± 23%	68% ± 21%
Colposcopy in expert hands	94% ± 6%	48% ± 23%
Full parameter fluorescence algorithm	82% ± 1.4%	68% ± 0%
Reduced parameter fluorescence algorithm	84% ± 1.5%	65% ± 2%

Pap: Papanicolaou.

Further improvements in diagnostic performance may be achieved by simultaneous measurement of tissue reflectance spectra. Diffuse reflectance spectroscopy (occasionally referred to as elastic scattering spectroscopy) is a simple, fast, and cost-effective method for probing tissue scattering and absorption characteristics. The optical properties of tissue vary spatially depending on its architecture, blood supply, and metabolic state; however, spatially resolved measurements of diffusely reflected light can be used to estimate tissue optical properties in vivo.[28] Several recent studies have suggested that differences in these optical properties, which were assessed using diffuse reflectance spectroscopy, can be used to discriminate normal from abnormal human tissues in vivo in the urinary bladder[29] and the skin.[30] Therefore, measuring both fluorescence and diffuse reflectance spectra may provide additional information of diagnostic value.

A system capable of measuring spatially resolved reflectance spectra and fluorescence excitation emission matrices in vivo would remove the limitations of many previous studies, potentially enabling the prediction of those excitation wavelengths that are able to provide the greatest discrimination between normal and abnormal tissues as well as a better understanding of the relative diagnostic potential of changes in the absorption, scattering, and fluorescence properties of tissue. To our knowledge, three such systems have been previously described in the literature.[31–33]

Richards-Kortum et al. recently developed a system for the in vivo measurement of fluorescence excitation emission matrices and spatially resolved diffuse reflectance spectra, and they described their work at the meeting to which this supplement refers. An arc lamp coupled to a scanning spectrometer provides continuously tunable excitation light that is coupled

into a fiber-optic probe. Resulting tissue fluorescence is collected through the fiber probe and delivered to an imaging spectrograph and a charge-coupled device (CCD) camera. Fluorescence emission spectra are collected sequentially at 20 excitation wavelengths, ranging from 290–480 nm, which then are assembled into a fluorescence EEM. Subsequently, white light is coupled into the probe and diffusely reflected light exiting the tissue at 3 spatial locations is detected from 320–900 nm using the spectrograph and CCD. Using this system, researchers can collect tissue EEMs and reflectance spectra with a signal-to-noise ratio in excess of 50:1 and 100:1 in approximately 2 minutes. This group of researchers currently is involved in a multisite, mid-sized clinical trial using the FastEEM technology to assess cervical fluorescence and reflectance spectra in 1800 women based on a clinical study design including statistically justified sample sizes and histopathologic analysis of directed biopsy as the gold standard.

Commercialization of Optical Spectroscopy for the Detection of Cervical Cancer

Based on the promising results of the initial algorithm, commercialization of the fluorescence technology was pursued by LifeSpex (Norcross, VA), a medical start-up company. The company has demonstrated the ability of their evoked tissue fluorescence technology to improve the colposcopist's ability to identify areas of clinically significant cancer precursors on the cervix. In a clinical investigation of prototypes of the handheld, full-field cervical imaging spectroscopy device at six sites in the U.S. and Canada, the LifeSpex device had a sensitivity of 88%, a specificity of 80%, and a negative predictive value of 97% for distinguishing HGSILs from non-SILs in 97 independent, prospective test subjects. When used as an adjunct to colposcopy, the device detected 15% more cases of HGSILs compared with colposcopy-directed biopsies in the 80 women with histologically proven HGSILs. Overall, the LifeSpex device detected 95% of cases with HGSILs. This in turn provides more effective diagnosis and treatment in patients with an abnormal Pap test result detected during routine practice.[34] In addition to LifeSpex, several other companies including SpectRx and Medispectra also have optical technologies for cervical detection under development. SpectRx has developed a multimodal hyperspectral imaging technology that probes both fluorescence and diffuse reflectance. In a study of 111 women scheduled to undergo colposcopy because of an abnormal Pap test result or other clinical finding, a first-generation prototype detected 25% more high-grade dysplasia (cervical intraepithelial neoplasia [CIN] of Grade 2 and greater) than did the Pap test by itself.[35] More recently, a new device that includes design features expected to be available in a commercial device has been tested by the company in greater than 400 patients at 4 medical centers, including the Medical College of Georgia; Emory University; St. Francis Hospital in Hartford, Connecticut; and the University of Miami Medical Center. Based on encouraging results, the company is building and testing a commercial device for upcoming Food and Drug Administration (FDA) pivotal trials.

To our knowledge, there currently is no commercially available or FDA-approved spectroscopic instrument for detecting cervical neoplasia. The reasons for this are manifold, including the regulatory climate, reimbursement issues, validation of diagnostic performance, changes in the competitive landscape, and balancing the cost versus feature equation. Thus far, the FDA has approved two fluorescence-based technologies, one for the detection of neoplasia in the lung and one for differentiating colorectal polyps. In addition, devices using other biophysical modalities are either on the market or currently under review.

Trimodal Spectroscopy

The modalities discussed so far have included fluorescence spectroscopy or the combination of fluorescence and diffuse reflectance spectroscopy. Further improvements in diagnostic performance may be achieved by simultaneously probing tissue fluorescence spectra, diffuse reflectance spectra, and the single scattering spectra of epithelial nuclei. Georgakoudi et al. developed a technology called trimodal spectroscopy (TMS) that appears to be effective in analyzing cervical SILs.[36] It has the advantage of providing three relevant types of medical information from which a combined diagnosis is obtained. Data are taken at the time of colposcopy using a FastEEM instrument that acquires a series of fluorescence spectra at 10 excitation wavelengths and a diffuse reflectance spectrum, all in a fraction of a second. Light is delivered and collected by means of a flexible optical contract probe. The spectra are analyzed by the following techniques: intrinsic fluorescence spectroscopy (IFS), diffuse reflectance spectroscopy (DRS), and light-scattering spectroscopy (LSS). IFS provides chemical information regarding the metabolic state of the tissue and the underlying collagen, DRS provides information regarding the scattering and absorption properties of the stroma, and LSS provides information regarding the enlargement of the cell nuclei in the epithelial layer. The information provided by these three techniques then is combined to form a diagnosis, with results in very good agreement with those of histopathology. The next step will be using the technique as a

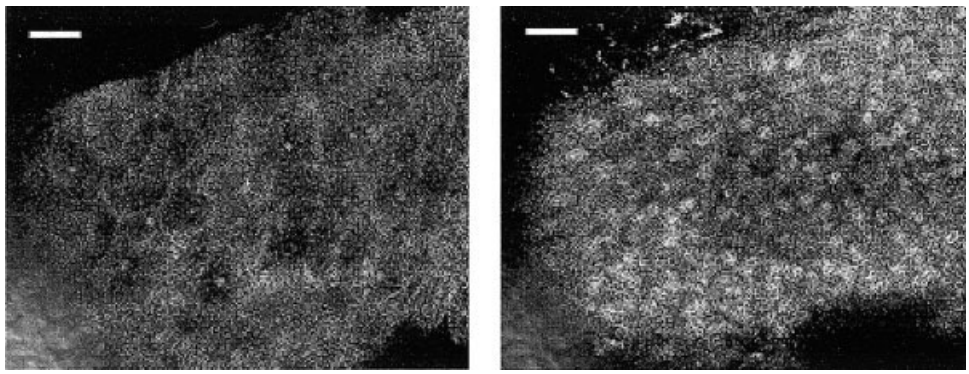

FIGURE 5. Confocal images illustrate normal and abnormal biopsy findings in a patient admitted for colposcopic examination. Compared with the image of normal tissue (left), the image of abnormal tissue (right) is characterized by increased nuclear size and density. Scale bar = 50 μm.

guide to biopsy to determine whether TMS diagnosis can improve biopsy yield. This requires the development of efficient software for real-time analysis of the spectral data, an area of active research (unpublished data). A further advance also under study by Gurjar et al. is the development of a wide-field spectral technique, based on LSS, that can image the entire cervix and indicate areas suspected of being cervical neoplasia.[37] The strategy is described as the spectral version of aceto-whitening because it provides a means with which to identify areas of potential abnormality. Areas identified as suspect then will be examined in more detail with the TMS optical fiber contact probe, which can sample a field measuring 1 mm in greatest dimension.

REFLECTANCE CONFOCAL MICROSCOPY

In addition to the optical technologies discussed so far that focus on probing the spectral information content of tissue, direct high-resolution imaging technologies also are currently under development. Precancerous cervical lesions are characterized by increased nuclear size, an increased nuclear/cytoplasmic ratio, hyperchromasia, and pleomorphism, which currently can be assessed only through invasive, painful biopsy. Screening and diagnosis could be vastly improved by optical technologies that image subcellular structure in vivo without the need for expensive and painful tissue removal, processing, and examination. In vivo confocal imaging is a new technology currently under development that provides the ability to noninvasively image cervical epithelial cells using reflected light.

In concept, in vivo confocal imaging is similar to histologic analysis of biopsy specimens, except that three-dimensional subcellular resolution is achieved without removing tissue, and contrast is provided without stains. A pinhole placed at a conjugate image plane isolates light returning from a finite volume, without the need for physical sectioning. Scanning the focal spot in the axial and radial dimensions forms an image of the reflectance values from the sample.

Collier et al. performed a study to assess the effect of a simple contrast agent–acetic acid (vinegar)–on confocal images of cervical biopsy specimens.[38] Using a nonfiber confocal microscope constructed in-house, researchers resolved subcellular detail throughout the entire cervical epithelial thickness. The addition of acetic acid was found to enhance the nuclear signal in all images, and the effect was observed seconds after its application. Images taken before application of the acetic acid on the precancerous biopsy specimen demonstrated increased reflectivity of both the cell membranes and the nuclei; in addition, the cells were more crowded and irregularly spaced. These differences were dramatically enhanced in the images taken after application of the acetic acid, and the images demonstrated an increased signal from the nuclei in both the normal and abnormal biopsy specimens. These results illustrate the hallmark differences between normal cervical epithelium and early neoplastic transformation and demonstrate the potential of this new technology, namely that quality information, similar to that obtained by histology, can be obtained without biopsy and expensive subsequent processing.

In another study, Collier et al. recently performed clinical trials comparing the diagnostic performance of confocal imaging with that of the current clinical tool, colposcopy (Fig. 5).[39] These trials further support the enormous potential of this new technology. Their results demonstrated that confocal imaging had a sensitivity of 89% and a specificity of 91%, which both were significantly better than those for colposcopy (a sensitivity of 85%, and a specificity of 69%). The increase in specificity is particularly significant; cost estimates of screening and detection for cervical precancerous lesions have demonstrated that the strongest

contributor to cost is the false-positive rate of colposcopy.[40] Furthermore, confocal images can provide point-of-care diagnostics, allowing combined diagnosis and therapy in a single see-and-treat office visit. This can further reduce health care costs and reduce the number of patients lost to follow-up.

OPTICAL COHERENCE TOMOGRAPHY

To image at depths within cervical tissue beyond the range of a confocal microscope, researchers must employ a different imaging approach known as optical coherence tomography (OCT). Although confocal microscopy can be used only to image tissue within a few hundred microns of the surface, OCT can yield backscattering data for depths of several millimeters. OCT has been shown to achieve resolutions in the cellular and subcellular range (range, 2–10 μm) and could improve the diagnostic capabilities of many clinical imaging procedures, including colposcopy. To evaluate OCT for the detection of those microstructural changes associated with cervical neoplasia in vivo, an integrated OCT colposcope was constructed by Pitris, who described this work at the conference discussed in this supplement to *Cancer*. This instrument permits the simultaneous en face viewing of structural features and allows precise registration of the OCT scan plane without interfering with normal medical procedures. The first clinical feasibility study resulted in the successful identification of neoplastic and microstructural changes. The use of image processing techniques can be a powerful method for analyzing OCT image data, especially when large numbers of patients are involved. Image processing allows complex image formation to be reduced to quantitative variables that can be statistically analyzed, quantified, interpreted, and used to predict the presence of disease. In addition, it enables faster and better coverage and visualization of large areas and large volumes of data.[41]

An example of such quantitative information from segmented OCT images is the extraction of statistical properties that describe tissue intensity. Preliminary results from Dr. Pitris appear to correlate regions of high intensities with areas of the most advanced neoplasia, and the application of OCT could be extended to large volumes of data.

Grading neoplasia most likely will require more information than the microstructural features resolved by standard resolution OCT. Cellular and even subcellular characteristics may be critical for the accurate determination of neoplastic grade. A very broad bandwidth Ti:Al2:O3 laser was used for the acquisition of ultrahigh-resolution OCT images. The system was comprised of a specially balanced and compensated interferometer and achromatic optics. Ex vivo ultrahigh-resolution OCT images of the normal cervix to our knowledge demonstrated for the first time the ability of OCT to delineate the presence of cells in human tissue. The squamous cells of the cervical epithelium, most likely with the presence of koilocytosis, are clearly evident in the images obtained. They range in size from approximately 10–30 μm. This finding implies that diagnostically useful cellular information can be extracted from human tissue using a high-resolution OCT system. Potential disadvantages of OCT include limitations in backscattering contrast between normal and dysplastic tissue, limited field of view, and limitations in imaging depth.

FUTURE DIRECTIONS

The current study summarizes current research aimed at developing effective optical technologies for detecting cervical neoplasia. A variety of technologies including both spectroscopic approaches (fluorescence spectroscopy, reflectance spectroscopy, and TMS) and direct imaging methods (confocal microscopy and OCT) currently are under development. These technologies are designed to address the detection and diagnosis of cervical precancerous lesions in the developing and developed world. In developing countries, critical needs include low-cost technology, low-maintenance equipment, and real-time diagnosis. In developed countries, cost reduction is a priority that can be achieved by avoiding overtreatment and identifying patient cohorts for see-and-treat strategies. Although numerous studies published to date have demonstrated the potential of optical technologies in small pilot studies, in order for these technologies to be translated successfully into clinical practice we need results from large multicenter trials, based on validated endpoints and conducted with validated equipment. Although preliminary studies have demonstrated the potential of optical spectroscopy and imaging to deliver sensitive and specific detection of precancerous conditions, to our knowledge the fundamental biophysical origins of variations in remitted optical signals between normal and neoplastic tissue have not been elucidated fully. We believe increased research efforts in this area are critical to exploit the potential of emerging optical technologies fully.

Finally, although optical technologies promise high-resolution, noninvasive functional imaging of tissue at competitive cost, to our knowledge, optical technologies currently probe only a limited number of endogenous chromophores. The combination of emerging optical technologies with the development of novel exogenous contrast agents, designed to probe the molecular specific signatures of cancer, would dramatically improve the detection limits and clinical

effectiveness of optical imaging. A number of exogenous contrast agents, ranging from targeted fluorescent dyes to particle-based materials (including quantum dots, nanoshells, and metal nanoparticles) currently are under assessment as potential contrast agents. Contrast agents may target a variety of markers of features such as altered differentiation, regulation, and proliferation. In particular, optical methods for in vivo human papillomavirus (HPV) detection and monitoring response to HPV vaccines will be important areas of future research. In addition, work must continue toward the development of optimal methods for the optical imaging of genetically induced contrast obtained via bioluminescence and/or green fluorescent protein.[42–44] In addition to the development of effective contrast agents, increased funding for the optical detection of cervical precancerous lesions for screening and diagnosis, funding for research networks, centers to standardize algorithms, and large diagnostic trials of various technologies would aid in the development and implementation of all these new optical technologies in the detection of cervical neoplasia and cancer and its precursors. Finally, all new research projects for emerging technologies should address the areas of technology assessment as outlined by Littenberg (biologic plausibility, technical feasibility, intermediate outcomes, patient outcomes, and societal outcomes) to ensure that only superior, not simply alternative, technologies are implemented in clinical situations.

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