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Optical Imaging for Cervical Cancer Detection: Solutions for a Continuing Global Problem

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Preface

Cervical cancer is the leading cause of cancer death for women in developing countries[1]. Optical technologies can improve the accuracy and availability of cervical cancer screening. For example, battery-powered digital cameras can obtain multi-spectral images of the entire cervix highlighting suspicious areas, and high-resolution optical technologies can further interrogate suspicious areas, providing *in vivo* diagnosis with high sensitivity and specificity. In addition, targeted contrast agents can highlight changes in biomarkers of cervical neoplasia. Such advances should provide a much needed global approach to cervical cancer prevention.

Cervical Cancer: A Global Challenge

Cervical cancer is the second most common cancer in women worldwide[1]; more than 80% of cervical cancers occur in the developing world where the least resources exist for management[1]. Most cases of cervical cancer can be prevented through screening programs, such as the Papanicolaou (Pap) smear aimed at detecting precancerous lesions for treatment. In countries in which organized programs have been established, the incidence and mortality of cervical cancer have dramatically reduced[2]. Yet, the necessary resources and infrastructure for screening are not available in many countries; as a result, 274,000 women die each year as a result of this preventable disease[1].

Cervical cancer is caused by chronic infection with high risk types of the human papilloma virus (HPV)[3]. The recent development of vaccines to prevent HPV infection promises to further reduce the incidence of cervical cancer in countries where vaccines are available[4]. However, the significant cost of vaccines and, in some cases, political or logistical barriers, could delay implementation of universal mass vaccination in many developing countries[5]. Furthermore, current vaccines are effective only against high-risk HPV types 16 and 18, which together account for approximately 70% of cervical cancers worldwide[5]. Because the vaccine does not cover all high-risk HPV subtypes, routine cervical cancer screening is still necessary, even for women who have been vaccinated, so cervical cancer screening remains necessary for the foreseeable future.

Clinical Approaches to Cervical Cancer Screening

Current screening and diagnosis of cervical precancer is based on optical techniques developed in the early 1900s[2]. As shown in the top row of Fig. 1, an abnormal Pap smear is followed by examination of the cervix using a low-power light microscope (colposcope) to visualize changes in tissue reflectance, which might indicate precancer. For decades, clinical investigators have searched for ways to improve the optical contrast between normal cervix and precancerous lesions. The use of simple agents such as acetic acid and Lugol's iodine, together with the use of a green illumination filter, can highlight suspicious regions. However, because the specificity of visual examination is low, colposcopically abnormal areas are routinely biopsied to confirm the presence of disease[6]. Implementing this approach requires

extensive infrastructure, personnel and economic resources; as a result, the vast majority of women in the world do not have access to life-saving screening programs[7].

Simple visual approaches have been explored to enable cervical cancer screening in resource-poor settings. For example, the use of visual inspection with acetic acid (VIA) is being explored as an alternative to Pap smear screening and colposcopy in many developing countries[7-10]. In VIA, a trained health care provider examines the cervix with the naked eye before and after application of acetic acid. Large clinical trials have been conducted to evaluate the performance of VIA for screening; Table 1 summarizes the findings of several of these trials. A recent review of the performance of VIA in six studies involving more than 65,000 women in South Africa, India, Zimbabwe, China, Burkina Faso, Congo, Guinea, Mali and Niger found that the sensitivity of VIA varied from 67-79% while specificity ranged from 49%-86%. The sensitivity of VIA is similar to that reported for Pap smear screening while specificity is lower, although some studies suffered from verification bias[11]. The use of low level magnification does not improve the performance of VIA appreciably[12, 13].

VIA has many advantages — it is inexpensive, requires minimal infrastructure and if abnormal areas are observed the patient can be referred for immediate treatment, circumventing the need for the expense and infrastructure of histology. However, because VIA relies on subjective visual interpretation, it is crucial to define consistent criteria for suspicious lesions and to train providers to correctly implement these criteria. Denny noted that restricting the definition of a positive VIA test to a well-defined acetowhite lesion significantly improved specificity, but reduced sensitivity[13]. In a series of 1,921 women screened in Peru, Jeronimo found that the VIA positivity rate dropped from 13.5% in the first few months to 4% during subsequent months of a two-year study; the drop was hypothesized to be due to a learning curve for the evaluator[10].

Recent advances in consumer electronics have led to inexpensive, high dynamic range charge-coupled device (CCD) cameras with excellent low light sensitivity. These technologies have been used to acquire digital images of the cervix in a relatively inexpensive way, with or without magnification[14]. Moreover, automated image diagnosis algorithms based on modern image processing techniques can assist and complement subjective visual interpretation[15-17]. These approaches, which we refer to as Digital Inspection with Acetic Acid (DIA), can potentially improve the performance of VIA.

Changes in Optical Properties

Both VIA and colposcopy rely on changes in the optical properties of neoplastic tissue to detect precancerous lesions. Image contrast between normal tissue and precancerous areas can be enhanced in a number of ways. Illuminating tissue with green light during colposcopy highlights the contrast associated with atypical blood vessels because hemoglobin absorbs green light. The application of acetic acid differentially increases the light scattering of neoplastic lesions[18], making them easier to visualize. While these simple approaches help clinicians recognize suspicious lesions, they exploit only a few of the changes in optical properties associated with the development of neoplasia. Recent studies have characterized a broad range of changes in tissue optical properties that occur with precancer[19-21]. Results of these studies are summarized in Fig. 2, and indicate that optical methods can be used to probe changes associated with known hallmarks of cancerous changes in tissue such as epithelial cell morphology, metabolic activity and differentiation[22], stromal angiogenesis[23, 24], and epithelial-stromal communication[25].

Light Scattering and Absorption

Optical technologies can interrogate changes in tissue architecture, cell morphology and biochemical composition. Most high grade precancers present with vascular changes due to the development of new blood vessels[26]. This angiogenesis can be seen visually, and quantified using image analysis approaches[17]. Hemoglobin has a characteristic absorption spectrum with peaks at 420 nm, 542 nm and 577 nm. Changing the wavelength of illumination can enhance vascular contrast and can probe vessels at different depths below the visual surface of the cervix. Acetic acid increases light scattering from cervical cell nuclei. Following application of acetic acid[18, 27], the mean scattering coefficient of precancerous tissue is approximately three times higher than that of normal epithelium[28, 29]. The difference in scattering between normal and precancerous epithelium is attributed to increased nuclear size, increased optical density of the nucleus and changes in chromatin texture[30, 31] that have been documented in cancerous cells. Finally, cervical precancer is associated with decreased stromal scattering, attributed to a degradation in collagen fibers possibly due to proteases secreted by pre-neoplastic epithelial cells[32].

Autofluorescence

The use of fluorescence interrogation can extend the range of biochemical changes which can be probed optically. In normal cervical tissue, collagen crosslinks give rise to bright fluorescence in the stroma over a broad range of excitation wavelengths [20, 33]. In normal women, this stromal fluorescence increases with age and with menopause[21], however, stromal fluorescence is greatly diminished in cervical precancers[20, 33] and cancers[34]. Cervical epithelial cells show cytoplasmic autofluorescence attributed to mitochondrial NADH using UV excitation wavelengths (~330-370 nm) and mitochondrial FAD using green excitation wavelengths (~510-550 nm) [20, 33]. In addition, cervical epithelial cells show autofluorescence at the periphery of the cells, often attributed to cytokeratins[28]. In normal epithelium, basal epithelial cells show strong cytoplasmic fluorescence; parabasal, intermediate and superficial cells showed fluorescence only at the periphery of the cell[20, 33]. Figure 2 compares confocal fluorescence images of organ cultures of normal human cervical tissue and precancer. In low-grade precancers, cytoplasmic fluorescence is visible in the bottom 1/3 of the epithelium and in high grade precancers, cytoplasmic fluorescence is visible throughout the lower two-thirds of the epithelium, with reduced fluorescence attributed to keratin[20, 33]. This is consistent with recent studies which show that HPV immortalized keratinocytes show increased NADH and FAD fluorescence relative to normal keratinocytes[35].

In Vivo Optical Imaging Technologies

In the last decade, advances in high-performance, low-cost electronics, have enabled development of sensitive systems for optical imaging and interrogation of cervical precancer *in vivo*[36-38]. Table 2 summarizes the capabilities of a variety of different optical interrogation methods under investigation. As illustrated in the bottom row of Fig. 1, these optical tools can be used to monitor biologically predictive features of cervical cancer, providing a global approach to detect cervical cancer which bridges the molecular, cellular, and tissue scales.

Multispectral Widefield Imaging

Widefield imaging relies on cameras to image changes in reflectance and autofluorescence at multiple wavelengths across the entire cervical epithelium. Typically, widefield imaging can achieve 50-100 micron spatial resolution, and can highlight suspicious regions of tissue. Point-probe techniques, which use a small fiber optic probe placed in contact with the tissue surface, can then be used to interrogate suspicious areas with higher spatial or spectral resolution.

A number of pilot studies have investigated features of multi-spectral reflectance imaging to assess which give rise to greatest image contrast. Reflectance imaging with green wavelength illumination consistently gives best contrast because of hemoglobin absorption[39]. Alternatively, color reflectance images obtained with white light illumination can be separated into red, green, and blue channels and analyzed to enhance image contrast[15, 40]. A pilot study of digital reflectance images of the cervix acquired from 29 women showed that an automated image analysis algorithm could identify the presence and spatial extent of high grade precancers with 79% sensitivity and 88% specificity compared to histopathologic analysis [15]. Illuminating tissue with light which has been passed through a linear polarizer while imaging reflectance through an orthogonally oriented linear polarizer can reduce specular reflection, bright areas caused by light reflected from the tissue surface, and improve visualization of subepithelial vascular patterns[39].

Imaging the time course of acetowhitening can also improve the ability to discriminate high grade precancer[39, 40]; a multispectral reflectance imaging study of 123 women found that the increase in light scattering after application of acetic acid was greater and persisted for a longer time in high grade precancers[41].

Similarly, tissue autofluorescence can be imaged in widefield mode[42]. Exciting autofluorescence in the UV and blue wavelengths (~440-470 nm) has been shown to give greatest contrast between normal tissue and precancer[42, 43]. Results of several large clinical studies investigating hyperspectral autofluorescence and reflectance imaging have recently been reported and are summarized in Table 1[44-47]. In these studies, autofluorescence and reflectance spectral data were collected with relatively high spectral (~5 nm) and spatial (~ 1 mm) resolution. Pattern recognition approaches were used to classify tissue and results were compared to the gold standard of histology to assess performance. In a study of 111 women, Ferris found a sensitivity of 97% and a specificity of 70% for hyperspectral widefield imaging compared to colposcopic directed biopsy or loop electro-surgical excision[46]. A larger series of 572 women were assessed with this device[47], yielding a sensitivity of 95.1% and a specificity of 55.2%. Huh and colleagues found similar results using a different hyperspectral imaging approach to detect UV induced fluorescence at 337 nm and reflectance in 604 women[44]. With a sensitivity of 92% and a specificity of 50%, they found that hyperspectral imaging could detect 1/3 more high grade precancers than colposcopy alone, with a relatively small increase in the false positive rate[44]. In a multi-center trial testing the device as an adjunct to colposcopy in 193 women, researchers found that the use of hyperspectral imaging resulted in a 22% relative gain in the true positive rate of colposcopy with an 18.1% incremental gain in the false positive rate[48]. A multi-center trial of this device involving 2,299 women randomized to receive colposcopy alone or colposcopy plus hyperspectral imaging showed similar results[45]. In addition, for women with a Pap smear showing atypical cells of uncertain significance or low grade precancer, hyperspectral imaging increased the true positive rate by 26.8% compared to colposcopy alone with a minimal increase in the false positive rate.

This device was approved by the US Food and Drug Administration in March of 2006 to enhance the sensitivity of colposcopic detection of high grade cervical precancers[49]. Thus, while widefield imaging can objectively detect cervical precancers with high sensitivity, specificity is limited. The use of other optical approaches to probe suspicious areas identified with widefield imaging may increase specificity. Two approaches which have been considered include point optical spectroscopy and high resolution optical imaging.

Optical Spectroscopy

Fiber optic probes can be used to record fluorescence and reflectance spectra of small areas of tissue with 1-5 nm spectral resolution, providing detailed, quantitative information about the distributions of optically active molecules within a tissue (Figure 3).

A number of studies have been carried out to assess whether optical spectroscopy can provide accurate *in vivo* diagnosis of cervical precancer; Table 1 summarizes results of key studies. Reflectance spectroscopy measures the intensity of light reflected as a function of illumination wavelength, providing information about changes in epithelial cell scattering, stromal scattering and stromal angiogenesis. Using empirical algorithms to classify tissue based on reflectance alone achieved sensitivity of 72% and specificity of 81% in one series of 161 patients[50], but approaches which use fluorescence spectroscopy alone or the combination of reflectance and fluorescence generally yield better classification accuracy[51, 52]. Much current effort is focused on the design of fiber optic probes[53, 54] and analysis strategies to separate reflectance signals from the epithelium and the stroma[32, 55] in an attempt to increase accuracy.

In vivo fluorescence spectroscopy can detect high grade precancer with good accuracy; early pilot studies focused on UV and blue excitation[56, 57]. More recently, a study of 146 patients comparing 18 different excitation wavelengths found that three broad ranges of excitation - 330-340 nm (UV), 350-380 nm (UV) and 400-450 nm (blue) excitation – gave best sensitivity and specificity for detection of high grade precancer[58]. Across all studies, fluorescence intensity of precancerous lesions is lower than that of normal squamous tissue, and the peak emission wavelength of precancers is shifted to longer emission wavelengths relative to that of normal tissue[56-59]. The decreased fluorescence intensity has been attributed to the decreased stromal fluorescence and increased stromal absorption of cervical precancers[60-62], while the spectral shift is attributed to both increased hemoglobin absorption and increased mitochondrial fluorescence in precancers[60-62]. Drezek showed that at 380 nm excitation, approximately 20% of detected fluorescence of squamous normal tissue is due to NADH, while 40-50% of detected fluorescence in high grade precancer is due to NADH[60]. Brookner found that the fluorescence of columnar normal tissue and metaplasia are lower than that of squamous normal tissue[63]. Since cervical precancers frequently develop at the junction between squamous and columnar epithelium, the performance of optical algorithms are often limited by the challenge of discriminating precancers at the squamo-columnar junction.

Several small studies have compared the performance of reflectance and fluorescence spectroscopy alone and in combination with reflectance spectroscopy for cervical precancer detection[51, 52, 64], with combined methods giving best results. Georgakoudi discovered that combining three modes of spectroscopy - fluorescence, reflectance, and light scattering - yielded better results than any individual mode[52]. Mirabal noted that reflectance spectroscopy could distinguish columnar normal from high grade dysplasia with higher specificity than fluorescence alone[50].

Importantly, Weingandt noted that inflammatory lesions may give rise to false positive fluorescence measurements[59], and recent studies in other organ sites indicate that inflammation and precancer both exhibit a similar loss in stromal autofluorescence[65]. Techniques which better probe changes in epithelial signatures, such as depth-resolved spectroscopy[54] or high resolution imaging[29, 66], may give rise to better specificity.

High Resolution Imaging

Small, flexible confocal microscopes have been developed to image cervical tissue and microfabrication techniques can be used to manufacture confocal microscopes with minimal power requirements. High resolution techniques can image tissue with sub-cellular resolution to probe changes in epithelial cell morphology and epithelial architecture without the need for biopsy, sectioning and staining[67] (Figure 3). Video-rate reflectance confocal microscopy yields images of intact epithelial tissue with 1-2 micron spatial resolution[68] and with the use of acetic acid, which increases nuclear scattering, determination of image parameters such as nuclear to cytoplasmic (N/C) ratio are possible[69]. Collier showed that the N/C ratio measured via confocal microscopy could separate high grade cervical precancers with a sensitivity and

specificity greater than 90%[29, 66]. Automated image analysis routines can be used to segment nuclei in confocal images of cervical tissue and objectively calculate N/C ratio[70]. More recently, fiber optic confocal microscopes have become available to acquire confocal images of cervical tissue *in vivo* at near video rate in both reflectance[71] and fluorescence modes[72]. While it is difficult to image weak autofluorescence *in vivo* using confocal fluorescence microscopy due to photobleaching limits, advances in optically active, targeted contrast agents can be used to tag biomarkers of interest with an optical signal which can be measured and quantified *in vivo*.

Contrast Agents for Molecular Imaging

Recent developments in confocal fluorescence imaging have shown the utility of new vital stains, such as IV administered fluorescein to highlight vascular changes and topically applied acriflavine to visualize cell nuclei[73]. In the last decade, enormous progress has been made to understand the molecular events that accompany carcinogenesis. Optically active, molecular-targeted contrast agents can be used to image these biomarkers *in vivo*[74, 75].

In general, targeted optical contrast agents consist of a probe molecule for molecular specific recognition of biomarkers conjugated to an optically interrogatable label[74]. Optically active contrast agents have been developed using antibodies[76] or peptides[77] to target biomarkers and using a number of different types of optically active labels, including metal nanoparticles[78, 79], quantum dots[80] and organic fluorescent dyes[76]. Fluorescent dyes conjugated to monoclonal antibodies provide a mechanism to target multiple cell surface receptors overexpressed on tumor cells, such as the epidermal growth factor receptor[76]. Alternatively, peptides such as the epidermal growth factor, can target receptors[77], yielding smaller molecular weight agents which provide an advantage for topical application. The broad excitation range and narrow emission spectra of quantum dots provides the ability to simultaneously image expression of multiple biomarkers[81, 82], although concerns exist about the cytotoxicity of these materials[83].

As an alternative, some contrast agents incorporate optically active metal nanoparticles[74, 78, 79, 82]. Gold and silver nanoparticles provide a strong source of backscattered light for contrast in widefield and high resolution imaging[84]; the scattering signal from a single nanoparticle has been shown to be equivalent to approximately 1 million fluorophores[85]. Unlike fluorescent dyes, metal nanoparticles are not susceptible to photobleaching, and gold is non-toxic and biocompatible[79]. Gold nanoparticles conjugated to anti-EGFR antibodies have been used to image cervical precancer *in vitro* with high contrast[78, 79]. EGFR overexpressing cells induce aggregation of gold nanoparticles, leading to non-linear enhancements in scattering which can magnify signal differences resulting from moderate levels of overexpression[78]. The aggregation-induced increase in signal yielded an image contrast ratio of 10-20 fold between images of normal and high grade cervical precancer labeled with anti-EGFR gold nanoparticles in one study, dramatically increasing contrast beyond values reported for antibody targeted fluorescent dyes[78].

Conclusions and Perspectives

New screening technologies should work for developed and developing countries. The decreasing incidence of cervical cancer in many developed countries is a testament to the impact of comprehensive screening programs[86]. Since current HPV vaccines do not prevent all cervical cancers, and since women in low resource areas may not have access to new vaccines for decades, we have incentive to continue developing low-cost, high-impact screening technologies that can continue to reduce the incidence of cervical cancer.

Optical imaging and spectroscopy can non-invasively assess the morphologic and biochemical changes associated with the development of precancer at the point-of-care. Driven by advances in consumer electronics, high quality optical images can now be obtained with low cost devices; tandem advances in digital signal processing provide the ability to automate image analysis. Thus, optical imaging is ideally suited for use as a screening technology. Results of large, multi-center trials of widefield hyperspectral imaging show that this approach has high sensitivity, but lower specificity. In addition, currently available imaging instrumentation is expensive and bulky, making it difficult to use in low-resource settings[87]; efforts to engineer lower cost, battery powered, portable devices are essential to support global translation. Further work is needed to improve specificity; efforts should focus on improving the ability to discriminate precancer from normal columnar tissue, metaplasia and inflammation.

Alternatively, depth-resolved spectroscopy or high resolution optical imaging may provide complementary information about optical changes in the epithelium, which can improve specificity. In particular, the sensitivity and specificity of high resolution *in vivo* imaging in pilot studies, coupled with recent developments in low cost, high resolution fluorescence imaging systems[88, 89] makes this approach especially appealing. However, large scale clinical trials are needed to confirm and optimize diagnostic performance of high resolution approaches. Harnessing the benefits of optically active targeted contrast agents to image cancer-related biomarkers may further aid performance.

As shown in Fig. 3, optical technologies provide a flexible approach to sample the full range of biochemical and morphologic changes which accompany precancer development. This multi-modal optical approach has the potential to improve the performance of precancer screening, and once appropriately validated, also has the potential to expand access to screening.

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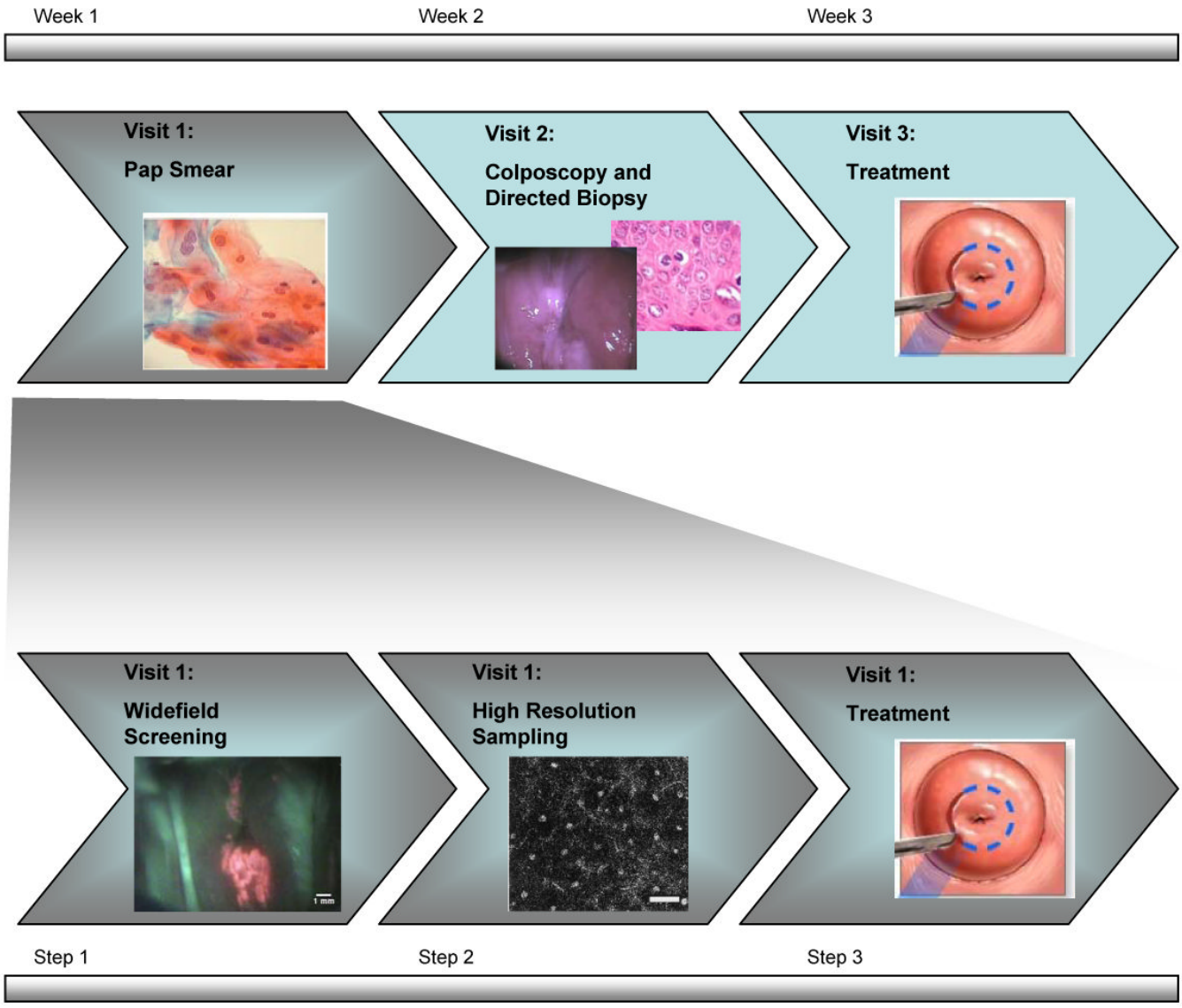

Figure 1.

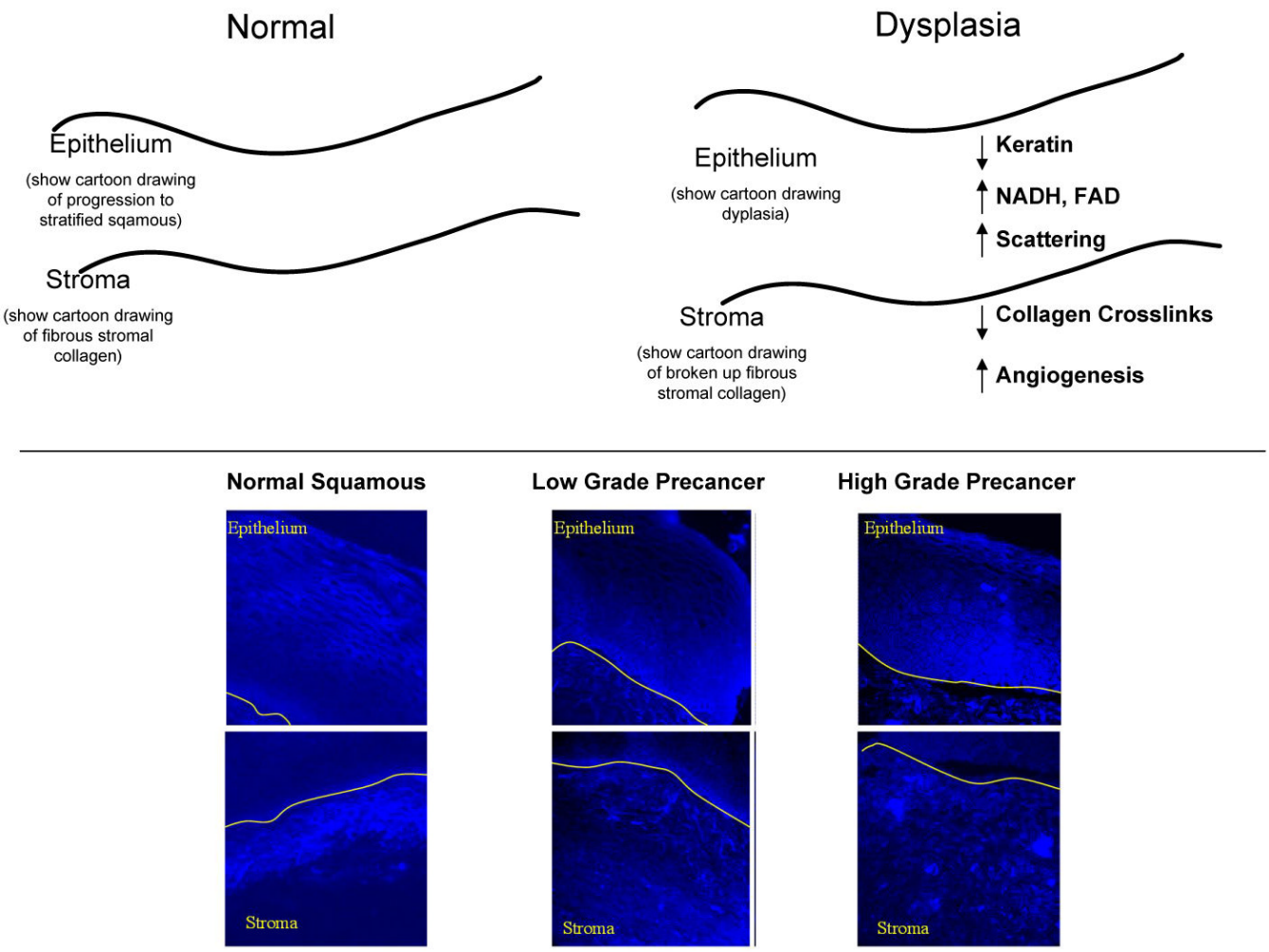

Figure 2.

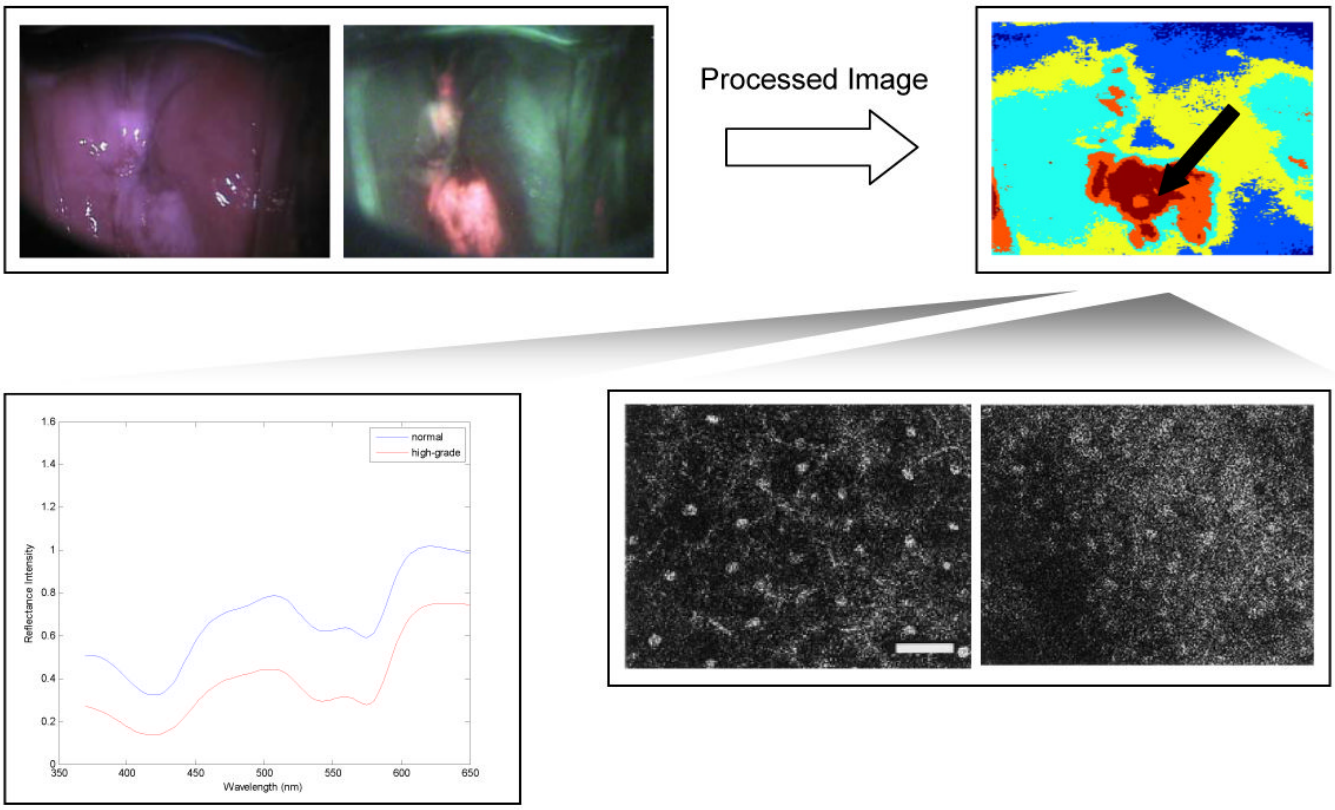

Figure 3.

Top row: Widefield reflectance and autofluorescence imaging can interrogate the entire field of the cervix, indicating suspicious areas; digital image analysis approaches can help to objectify and automate the recognition of abnormal areas with high sensitivity. High spectral or spatial resolution techniques can be used to probe suspicious areas to confirm diagnosis of precancerous areas. Spectroscopy (lower left) can probe changes in the concentration of tissue chromophores, while confocal microscopy (lower right) can directly images changes in cell morphology and nuclear to cytoplasmic ratio without the need to biopsy, section and stain tissue. Scale bars measure 1mm in the widefield images, and 50 um in the confocal images.

Table 1

Recent clinical trials (top) and pilot studies (bottom) testing *in vivo* optical imaging techniques for early detection of cervical cancer and its precursors

	Type of Detection	N = # of Patients in Analysis	Sensitivity (%) / Specificity (%)	Type of Study
Major Trials	Colposcopy[6]	5378	85/69	Prospective
	VIA[90]	2148	77/64	Prospective
	VIA[91]	2817	67/83*	Prospective
	VIA[92]	1997	71/74*	Prospective
	VIA[13]	2575	70/79	Prospective
	VIA[93]	1093	79/49	Prospective
	VIA[94]	54981	79/86	Prospective
	Widefield[44]	604	92/50	Prospective
	Widefield[46]	111	97/70*	Prospective
	Widefield[47]	572	95/55	Prospective
Pilot Studies	Spectroscopy[51]	161	83/80	Cross-Validation
	Spectroscopy[52]	44	92/90	Cross-Validation
	High Resolution[29]	28‡	100/100	Prospective
	High Resolution[66]	38‡	100/91	Retrospective

Visual Inspection with Acetic Acid (VIA)

*The threshold for positive test result is LGD. For all others the threshold is HGD.

‡N = # of biopsies and not the number of patients.

Table 2

Overview of optical technologies being used for the detection of cervical cancer.

Technique	Spatial Resolution	Field of View	Depth	Sources of contrast	Cost	In clinical use?
Visual Inspection	100u-200u	Entire cervix	Surface	Induced change in scattering	$	Yes
Widefield Imaging	50-100μ	Entire cervix	Surface	Fuorescence: Collagen Reflectance: Hemaglobin absorption, Acetic acid induced change in scattering	$$-$$$	Yes
Spectroscopy	1mm	1mm	.3-1mm	Fuorescence: Collagen, NADH, FAD Reflectance: Hemaglobin absorption, morphologic changes, DNA content, chromatin texture	$$	No
High Resolution Imaging*	1-2μm	<1mm	<1mm	Fluorescence: Fluorescently labeled probes Reflectance: Acetic acid induced change in scattering, morphologic changes, scattering coefficient	$$$	No

$, <$100; $$, $5,000-$30,000; $$$, >$30,000 (in U.S. Dollars)

*Laser scanning confocal microscopy