Confocal Laser Endomicroscopy
A Primer for Pathologists

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Context.—The advent of new endoscopic optical techniques is likely to change pathologists’ role in diagnosis.

Objective.—To describe how confocal laser endomicroscopy (CLE) works, show its advantages and limitations compared to cytohistologic biopsy, and explore how it may affect the practice of pathology.

Data Sources.—Literature review.

Conclusions.—Confocal laser endomicroscopy is proving its ability to provide histology-like images of tissues in vivo to help avoid risks and costs of conventional biopsies. Confocal imaging restricts light to 1 plane, emulating a paraffin section, and topical or systemic optical contrast agents allow subcellular resolution. New contrast agents could theoretically permit molecular characterization. In vivo imaging has begun to demonstrate novel, dynamic types of diagnostic features. Decreased histologic biopsies can be anticipated for a few scenarios. Significant limitations of CLE include the inability to create a tissue archive for broad molecular classification, suboptimal contrast agents, small fields of view and shallow penetration, paucity of clinical validation studies, and problems with reimbursement. Confocal laser endomicroscopy exposes new opportunities for pathologists: CLE technologies can be exploited in pathology, and diagnostic criteria expanded based on endoscopists’ discoveries. Potential synergy exists between CLE and cytology, allowing the low-magnification diagnostic architectural changes by CLE and cytology to emulate the full diagnostic information in a histologic biopsy while providing an archive of material for molecular or immunohistochemical studies. Confocal laser endomicroscopy will decrease some types of biopsies, but offers an opportunity for pathologists to find new ways to provide value and improve patient care.


Confocal Laser Endomicroscopy

A NEW ENDOSCOPIC TECHNOLOGY

Confocal laser endomicroscopy (CLE) is a recent endoscopic advance with promise to help circumvent these problems. Confocal laser endomicroscopy allows high-resolution histologic analysis of targeted tissue, or “optical biopsies,” in vivo and in real time during endoscopy.5,6

There are 2 Food and Drug Administration (FDA)—cleared platforms for CLE. Initially designed in 2004, endoscope-based CLE (eCLE; Pentax Corporation, Montvale, New Jersey) uses a fiber-optic cable to convey blue laser light to a miniaturized confocal microscope integrated into the 12-mm-diameter tip of an endoscope.2 Another platform, probe-based CLE (pCLE; Mauna Kea Technologies,6 Newtown, Pennsylvania) uses a fiber-optic probe bundle to convey light from a confocal microscope situated outside the patient to a port in a standard endoscope.2 Both platforms use a pinhole-like benchtop confocal microscope, to exclude light from planes above and below a plane of interest. Thus, CLE allows for an optical section to be observed, somewhat analogous to a histologic tissue section, without detection of out-of-plane light. Most current applications use a gray scale and collect only 1 color. Images from adjacent planes can be blended to create a 3-dimensional image.10
The Table compares the resolution and properties of eCLE and pCLE. Note that the axial resolution (i.e. perpendicular to the mucosa) is necessarily less than the lateral resolution (tangential to the mucosa) because of the physical properties of confocal microscopes. Thus, images reconstructed in the perpendicular orientation preferred by pathologists will look relatively blurry compared to tangential optical sections. Endoscope-based CLE has improved lateral and axial resolution compared to pCLE, with the ability to adjust depth of focus below the mucosal surface up to a depth of about 250 μm. However, eCLE is considerably bulkier than pCLE, and thus pCLE is finding applications in smaller-sized spaces. A recent modification of pCLE decreases the diameter of the probe to 350 μm, allowing it to fit within a 22-gauge needle and provide a 300-μm field of view (about two-thirds of a ×400 microscope field). Endoscopic ultrasound guidance can then allow the probe to be inserted through a visceral organ into solid tissues/masses, analogous to fine-needle aspiration (FNA) sampling. Using a porcine model, liver, pancreas, spleen, and lymph nodes have been directly visualized with needle-based pCLE.11

**FLUORESCENT DYES FOR CLE**

Confocal laser endomicroscopy relies on fluorescence. In most contexts, tissue autofluorescence does not provide sufficient contrast. Exogenous contrast agents can be applied topically or intravenously. Five milliliters of intravenous 10% fluorescein (Fluorescite; Alcon, Hänenberg, Switzerland) has been widely used in CLE. Intravenous fluorescein is only FDA approved for retinal angiography, but it has found widespread off-label use in CLE. Fluorescein is chemically closely related to eosin and they produce essentially identical staining of cytoplasm and extracellular matrix. Fluorescein also highlights the structure and physiology of the vasculature in a manner relatively unfamiliar to pathologists: it shows vessel density and shapes, it accumulates near leaky capillaries, and it accumulates in the lumen in areas of ulceration. Like eosin, it provides no direct visualization of nuclei, though the relative absence of fluorescein in the nuclei can allow some estimate of nuclear size and position.5,10,13,14

Nuclear staining can be achieved by the use of 0.05% topical acriflavine hydrochloride (Sigma Aldrich, St Louis, Missouri). Acriflavine was originally used in the early 20th century as an antimalarial and antibacterial drug, with little evidence of toxicity in humans. Acriflavine binds to nucleic acids, is potentially mutagenic, and is not FDA approved for any application. Nevertheless, numerous human clinical studies, primarily in Europe, have documented the usefulness of topical acriflavine in gastrointestinal endoscopy. To a pathologist accustomed to hematoxylin-eosin staining, acriflavine-stained tissue images can be inverted to have a white background with dark nuclear staining. To give an example of the resolution of eCLE as it compares to standard histology, Figure 1 shows a villous adenoma in a mouse model, stained with topical acriflavine and pseudocolored to resemble hematoxylin staining. Besides concerns about mutagenicity, acriflavine has limited penetration into tissue, and gives relatively uneven staining.

Cresyl violet (Sigma Aldrich) has the advantage of being fluorescent while producing a useful staining pattern like methylene blue with white light. Methylene blue is familiar to endoscopists because it is used in chromoendoscopy. Chromoendoscopy uses only magnification of reflected light from surfaces, with the methylene blue or cresyl violet staining helping to disclose surface changes characteristic of neoplasia. Fluorescence from the cresyl violet allows confocal optical sectioning into the mucosa, and it provides a cytoplasmic stain roughly similar to eosin or fluorescein. Compared to intravenous fluorescein, topical cresyl violet does not demonstrate vascular structure and function. Goetz et al19 studied the simultaneous use of topical 0.13% cresyl violet chromoendoscopy with subsequent eCLE in the lower gastrointestinal tract and appreciated negative nuclear contrast staining as cresyl violet enhanced the cellular cytoplasm. Of 57 lesions available for analysis from 36 patients, nonneoplastic and neoplastic changes based on architecture and nuclear size were identified with high interobserver agreement, with a positive and negative predictive value of 89%, 100%, and 100%, respectively (Figure 2; eCLE image).19

Fluorescent probes specific to biomarkers of disease are being investigated for use with CLE. Hsiung et al19 used a phage display library to identify a 7-amino-acid peptide that specifically recognized the surface of adenomatous colonic cells. The fluoresceinated peptide was then used to topically label the colonic mucosa, and by CLE, it bound more strongly to dysplastic colonocytes than to normal cells, with 81% sensitivity and 82% specificity. Other optical probes for molecular marker detection by CLE include fluorophore-conjugated and gold-nanoparticle-conjugated monoclonal antibodies.20

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**Specifications of Probe-Based (pCLE) and Standard Endoscope-Based Confocal Laser Microscopy (eCLE) and Conventional Video Endoscopy Equipment**

<table>
<thead>
<tr>
<th></th>
<th>eCLEa</th>
<th>pCLEb</th>
<th>Miniprobe, Needle-Based</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal diameter, mm</td>
<td>Mostly Upper and Lower GI</td>
<td>Upper and Lower GI</td>
<td>Biliary</td>
</tr>
<tr>
<td>Field of view, μm</td>
<td>475</td>
<td>240 or 600</td>
<td>320</td>
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<tr>
<td>Imaging depth, μm</td>
<td>0–250</td>
<td>70–130</td>
<td>40–70</td>
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<tr>
<td>Lateral resolution, μm</td>
<td>0.7</td>
<td>1–3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Axial resolution, μm</td>
<td>7</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Image rate, images/s</td>
<td>0.8–1.6</td>
<td>9–12</td>
<td>9–12</td>
</tr>
</tbody>
</table>

Abbreviation: GI, gastrointestinal tract.

a Pentax Corporation, Montvale, New Jersey.7
b Mauna Kea Technologies, Newtown, Pennsylvania.4
POTENTIAL CLINICAL APPLICATIONS: GASTROINTESTINAL

Colorectal Disease

The majority of recent clinical studies assessing CLE’s diagnostic potential have involved the gastrointestinal tract. Kiesslich et al.\(^\text{15}\) performed the first prospective clinical human trial with eCLE in 2004 and developed an eCLE classification for colorectal cancer screening. Their classification scheme, based on crypt, cellular, and vessel architecture, showed high accuracy for eCLE diagnosis of adenomatous changes compared to normal tissue in 42 patients with 134 lesions (sensitivity, specificity, and accuracy: 97.4%, 99.4%, and 99.2%, respectively).\(^\text{15}\)

Another study of 153 ulcerative colitis patients undergoing surveillance colonoscopy found, compared to conventional colonoscopy, that the combination of chromoendoscopy with eCLE was able to achieve the same rate of neoplastic lesion pickup with half the number of biopsies (decreasing from about 40 to 20 biopsies per patient).\(^\text{21}\) The accuracy of eCLE in distinguishing neoplastic from benign and regenerative foci was 98.3%.\(^\text{21}\)

Confocal laser endomicroscopy has been shown to specifically detect lymphocytic and collagenous colitis in the evaluation of chronic diarrhea, including observation of mononuclear infiltration in lymphocytic colitis and subepithelial collagenous bands in collagenous colitis.\(^\text{22–24}\)

Barrett Esophagus

Standard surveillance for esophageal dysplasia and adenocarcinoma in Barrett esophagus patients entails 4-quadrant biopsies every 1–2 cm for the length of Barrett...
Endoscope-based CLE has been shown to accurately differentiate normal epithelium from metaplasia and dysplasia. Using intravenous fluorescein, a "confocal Barrett classification" was proposed based on capillary distribution and leakiness of fluorescein, the presence of goblet cells, and enlarged nonstaining holes representing enlargement of nuclei. Compared to histologic biopsy, the classification had a sensitivity and specificity of 98.1% and 94.1% for detecting Barrett mucosa, and sensitivity and specificity of 92.9% and 98.4% for detecting low- or high-grade dysplasia.

To confirm the classification and to compare efficacy of eCLE-targeted biopsies to standard 4-quadrant surveillance biopsies, a small, prospective, crossover, double-blind study randomized 39 Barrett esophagus patients to eCLE-targeted biopsy and standard surveillance biopsies. Half as many eCLE-targeted biopsies (10 fewer per patient) detected the same number of high-grade lesions as standard surveillance biopsies. The theme that emerges from this and the colonic findings is that CLE has value in decreasing the number of biopsies required. High interobserver agreement has been achieved for diagnosis of dysplasia by CLE in Barrett esophagus, with a k value of 0.83.

**Gastric Cancer/Gastritis/Small Intestine**

Based on previously described gastric pit and vasculature patterns, gland configurations, and altered cellular architecture, CLE is able to distinguish gastric cancer and gastritis and detect Helicobacter with high accuracy. A blinded prospective study of 132 patients assessing gastric pit patterns showed sensitivity of 90.0%, specificity of 99.4%, and accuracy of 97.1% for gastric cancer. In vivo detection of villous atrophy and intraepithelial lymphocytes in celiac disease has also been described.

**Hepatobiliary/Pancreas**

Standard histologic biopsy has a low sensitivity for detecting cholangiocarcinoma. Based on the microvascular pattern, pCLE during endoscopic retrograde choanalgiopancreatography detected cholangiocarcinoma with sensitivity of 83%, specificity of 88%, and accuracy of 86% in 14 patients with biliary strictures compared to 50%, 100%, and 79%, respectively for histologic biopsy. The authors did not compare the sensitivity to biliary brush cytology.

Using a rigid pCLE during laparoscopy of 25 patients with liver disease, subsurface images described liver cellular anatomy and pathology with good correlation to biopsies. Finally, a recent case report used pCLE to detect villous structures of intraductal papillary mucinous tumors of the pancreas.

**POTENTIAL CLINICAL APPLICATIONS: OUTSIDE THE GASTRO INTESTINAL TRACT**

**Pulmonary**

Current bronchoscopy has been unable to access terminal respiratory units including bronchioles smaller than 3 mm. Recent studies with pCLE have been able to image and characterize these distal lung components including alveolar ducts and alveoli, except for apical and posterior upper lung segments. Using pCLE, autofluorescent patterns allowed a distinction between normal, premalignant, and malignant processes in peripheral lung nodules.

**Urinary/Gynecologic**

In the first pCLE study of the genitourinary tract using intravenous and intravesicular fluorescein with a rigid cystoscope, differences between normal urothelium and low- to high-grade tumors were appreciated; however, the rigid cystoscope was unable to access the anterior bladder wall for imaging or to maintain fluorescein image quality.

A prospective study of 15 patients undergoing colposcopy and loop electrosurgical excision procedure for previously diagnosed cervical intraepithelial neoplasia (CIN) evaluated the diagnostic yield of CLE for CIN detection. The study showed an overall sensitivity of 97% for detecting early squamous epithelial changes with a specificity of 80% for detecting normal or CIN1 and 93% for detecting CIN2 or CIN3. Confocal laser endomicroscopy offers potential to decrease the need for histologic biopsies prior to a "see and treat" loop electrosurgical excision procedure at the colposcopy following an abnormal Papanicolaou test result.

**Physiology/Therapy**

As CLE offers real-time, in vivo visualization, the optical tool has the potential to observe cellular structure and functions over time. Dynamic changes in structure will provide new insights into physiologies at the bedside. Visualization of diagnostic altered vascular physiologies was alluded to above. Apoptosis of hepatocytes was recently observed in vivo in live mice during 240 minutes of observation time. Responses to antineoplastic therapies could theoretically be measured over time in vivo at a cellular level. An interesting dynamic feature, not evident in standard histologic sections, has been described using CLE: the closing of the gaps in the epithelium left by shedding intestinal epithelial cells. Defects in the closing, with bacterial invasion of the gaps, are observable in inflammatory bowel disease patients by CLE, a finding with major pathophysiologic significance.

**LIMITATIONS OF CONFOCAL LASER ENDOMICROSCOPY**

The immediate applications for CLE appear to be to safely decrease the number of biopsies required for cancer detection in specific scenarios (eg, ulcerative colitis and Barrett esophagus surveillance, or possibly pre–loop electrosurgical excision procedure for patients with abnormal Papanicolaou test results). CLE may be able to eliminate the need for small biopsies for certain nonneoplastic diseases (eg, collagenous colitis). However, a number of practical and technical obstacles severely limit the ability of CLE to replace the need for pathologists in other small biopsy interpretations.

The biggest limitation of CLE is that it does not provide an archive of tissue for full molecular characterization. A poorly differentiated tumor could possibly be diagnosed, but not likely classified, and prognostic or therapeutic markers cannot probably be tested in the foreseeable future in vivo by CLE. The prospect of molecular diagnostic imaging, that is, using labeled peptides or antibodies to detect particular biomolecules or antigens, faces tough challenges. Optical labels must be able to diffuse through tissue if applied topicaly, and the label...
must be able to penetrate cell membranes if the target is intracellular. To be able to detect a significant molecular pathway implies the potential to either disrupt or augment the pathway by the optical imaging agent, and thus it is likely that the method for detecting each and every molecule would be subject to considerable FDA scrutiny. Confocal laser endomicroscopy could be extended to include more than 1 color or 1 biomolecule measurement at a time, but one cannot often predict ahead of the procedure which biomolecules need to be assessed.

Another physical limitation is that CLE cannot currently penetrate beyond the mucosa of the gastrointestinal tract, preventing diagnosis of submucosal invasion. Thus, the ability to perform immediate mucosal resections is hampered. Depth of penetration (0.25 mm) could be minimally enhanced by 2 photon techniques, especially if new infrared fluorophores were developed and proven to be safe in vivo. Increased penetration necessarily means decreased lateral resolution with current technology. Although concurrent fine needle-sized pCLE probes theoretically offer the potential for submucosal staging, there would not seem to be much of an advantage of using needle-based CLE over other minimally invasive micro-biopsy techniques.

Confocal laser endomicroscopy is FDA cleared, but the Centers for Medicare and Medicaid Services have not established a Current Procedural Terminology code for its use, and thus reimbursement is probably at the level of conventional endoscopy. Confocal laser endomicroscopy technology is expensive, and it is difficult to envision cheaper solutions. Low-cost high-resolution fluorescent imaging systems (consisting of just a bundle of micrometer-scale fiber-optic cables that transmit a one-to-one indexed image to a digital camera) have been described, but these cannot achieve optical sectioning or visualize structures more than a few micrometers below the surface. Optical sectioning via confocal or 2-photon technologies is needed to exclude photons from above and below the plane of interest to prevent degradation of image quality.

There are not strong clinical outcome measures to justify Centers for Medicare and Medicaid Services reimbursement of CLE at a higher level. It is not obvious, for example, that increased detection of dysplasia in microscopic foci detected by CLE would improve patient outcomes. A better rationale for reimbursing CLE at a higher level may be that it appears to safely reduce the number of biopsies needed for surveillance. The data of Dunbar et al. suggest that its added value for Barrett esophagus patients compared to conventional endoscopy could be as high as the charges for 10 endoscopic biopsies. The data of Kiesslich et al. suggest that 70 CLE images can substitute for about 30 endoscopic biopsies for cancer surveillance in ulcerative colitis. Data on the time required for clinicians to perform CLE are mixed. In the previously described study of cancer surveillance for ulcerative colitis, in which patients were randomized between conventional endoscopy and chromoendoscopy plus eCLE, the average time for conventional endoscopy (31 minutes; range 18–48) was surprisingly not statistically longer than the time (by endoscopists highly experienced in CLE) for combined chromoendoscopy plus eCLE (42 minutes; range, 29–64 minutes), even though an average of 70 CLE images were collected for each patient. On the other hand, a rough estimate showed that CLE added about 18 minutes to the standard endoscopy time for Barrett surveillance. The increase was partially offset by about 7 minutes because of decreased numbers of histologic biopsies when CLE is used.

**FUTURE OF CONFOCAL LASER ENDOMICROSCOPY: TURF WARS, OR NEW OPPORTUNITIES FOR PATHOLOGISTS?**

Until further advances in CLE allow cellular- and subcellular-level morphology to be fully discerned, CLE is somewhat analogous to a low-magnification tissue section. As such, it complements cytology. One can envision using the tissues’ architectural information provided by CLE in conjunction with cytology brushings to achieve better sampling, to achieve a synergistic diagnostic accuracy surpassing that of either CLE or histologic biopsy alone, and with a safety surpassing histologic biopsy. Cytology also synergizes with CLE by providing the needed archive for molecular or immunohistochemical studies.

Does CLE really have any advantage over cytology? Mucosal evaluation by CLE is restricted to a depth that is comparable to or less than minimally invasive cytology brushings. Confocal laser endomicroscopy typically has a field of view that is close to the diameter of the tissue fragments that can be obtained by FNA (eg, less than 500 μm, or one ×400 microscopy field). With hematoxylin and eosin staining of paraffin-embedded tissue, these field dimensions are sufficient to allow invasion to be diagnosed in efficient cell blocks of breast FNAs. With improvements in the speed and efficiency of recovery of FNA microbiopsy fragments for paraffin embedding and sectioning and with improvements in microbiopsy needle design, there would not seem to be much of an advantage in using the new needle-based pCLE system for diagnosing solid organs. Needle-based pCLE is at least as invasive as FNA, needle-based CLE still lacks optical contrast agents to bring out diagnostic or molecular features, and it cannot provide an archive of tissue.

The technological achievements that will be required for endoscopists to image more deeply into tissue (for example, with 2-photon microscopy or infrared fluorescent imaging) could be even more useful for pathologists than they are for endoscopists. As an example, paraffin embedding and sectioning might not be needed if ex vivo biopsies were able to be immediately optically sectioned at the bedside in a biopsy cup. The future of CLE may be to allow accurate targeting of the smallest possible biopsy for immediate ex vivo bedside analysis of unprocessed biopsies. Such ex vivo biopsy fragments could be rotated to an appropriate plane perpendicular to the mucosa, improving resolution compared to in vivo confocal microscopy. Ex vivo microbiopsy fragments could also be subject to a myriad of fluorescent stains that cannot be used in vivo. Finally, microbiopsy fragments, after bedside diagnosis, could be appropriately triaged for molecular characterization. These applications are better suited to the skill set of pathologists than to that of endoscopists.

The difficulty of implementing new optical probes for CLE makes it hard for CLE to keep pace with molecular advances in pathology. New molecular or immunohistochemical markers that prove clinically useful in pathology are likely to be difficult to incorporate into CLE practice.
because of the need for complex safety evaluations. The upper- and the lower-GI tract. PM. A fluorescence confocal endomicroscope for in vivo microscopy of the G797–G806. We provide value and improve patient care. References


