O ptical imaging is a rapidly evolving field that encompasses several techniques that can be used for noninvasive imaging of biologic tissue specimens. Unlike other types of imaging, this modality uses light in the visible spectrum and harnesses the special properties of photons to obtain detailed images of tissue without exposure to harmful radiation. Optical imaging platforms are therefore safer, faster, and, most importantly, well suited for repeated procedures over time. Recently, advancements in biophotonics, computer science, instrumentation, and technology have aided in the development of several types of optical imaging platforms, many of which are now available for biomedical applications. Techniques such as confocal laser endomicroscopy and optical coherence tomography allow high-resolution, real-time in vivo imaging of luminal organs, enabling early detection of mucosal alterations. In addition, these imaging techniques also facilitate the procurement of targeted biopsies for better categorization of mucosal changes.

Unlike in vivo optical imaging, ex vivo optical imaging is not yet routinely used in clinical practice, although several optical imaging modalities are available for ex vivo tissue examination. These techniques include full-field optical coherence tomography, confocal fluorescence microscopy (CFM), and multiphoton microscopy. These optical imaging modalities have been widely used for ex vivo imaging in research studies, but there is limited evidence regarding the feasibility of their use in routine surgical pathology practice. The availability of these platforms for ex vivo tissue specimen imaging provides the opportunity for establishing the feasibility of using these platforms in surgical pathology laboratories and investigating potential applications in surgical pathology practice.

In this pilot study, we tested the feasibility of using a CFM platform for ex vivo imaging of tissue specimens obtained from surgical resections. The primary objectives of our study were to test the ability of the CFM platform to generate images of the sampled tissues and to compare the images with hematoxylin-eosin (H&E)–stained tissue sections for recognition of the imaged tissue.

MATERIALS AND METHODS

This prospective study was conducted at The University of Texas MD Anderson Cancer Center, Houston, using residual tissue fragments. The authors have no relevant financial interest in the products or companies described in this article.

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specimens obtained from surgical resections that had been accessioned in the surgical pathology laboratory for routine gross and microscopic pathologic examination. The present study was approved by the MD Anderson Institutional Review Board with a waiver of informed consent.

Fragments of fresh tissue measuring from 0.5 × 0.5 cm to 1.5 × 1.5 cm with thickness varying from 0.2 cm to 0.5 cm were collected from normal as well as areas of tumor from a total of 55 surgical resections that were performed for malignant tumors of the breast, liver, lung, and kidney soon after completion of immediate intraoperative assessment of the surgical specimens. Specimens were kept moist in isotonic phosphate-buffered saline (pH 7.4) prior to imaging. Imaging was performed within 1 hour of tissue procurement. The fresh tissue specimens were stained with 0.6 mM acridine orange for 1 minute to stain the nucleus and create the necessary contrast for recognition of tissue architecture. Acridine orange is a fluorescent dye that binds preferentially to nuclear DNA and has been used as a contrast agent in previous CFM studies. Excess dye was dabbed from the stained tissue with tissue gauze, and the specimen was immediately imaged.

The CFM images of the specimen were obtained using a confocal scanning microscope (Vivascope 2500, Caliber Imaging and Diagnostics Inc, Rochester, New York) designed specifically for ex vivo imaging of fresh biologic tissue specimens. Details of the optical design of the confocal microscope used in the study have been described previously.21 The system includes a diode laser that operates at a wavelength of 488 nm, a 550-nm bandpass filter, maximum illumination power of 5 mW, and a 30X water immersion objective lens with a numerical aperture of 0.9. At these settings, the lateral resolution was 1.0 μm; axial resolution was less than 5.0 μm. Images were acquired at a frame rate of 9 frames per second. The tissue specimen was placed in the stage of the microscope; covered with optical glass, which compressed the tissue; and scanned from one end to the other. Images of all scanned areas were captured as square confocal images measuring 630 × 630 μm that were stitched together to create a mosaic of images of the entire tissue. The resulting composite image measured up to 2.0 cm in greatest diameter. Composite images were captured at 2 different axial depths, including one from the surface and the other at a depth of 200 μm beneath the surface of the tissue.

After completion of imaging, the tissue was immediately fixed in 10% neutral-buffered formalin, subjected to routine processing, and embedded in paraffin wax to generate tissue blocks. The formalin-fixed and paraffin-embedded tissue blocks were cut at 5-μm thickness and stained by the H&E method for conventional histopathologic examination.

The composite CFM images for each specimen were examined in detail at different magnifications by the principal investigator of the study (S.K., a board-certified senior pathologist) for recognition of the tissue in conjunction with conventional histopathologic examination of the H&E tissue sections obtained from the imaged tissue.

RESULTS

We imaged 55 tissue specimens that were obtained from surgical resections of the breast (16; 29%), lung (18; 33%), kidney (14; 25%), and liver (7; 13%). Mosaics of grayscale CFM images were acquired in 5 to 10 minutes. Interpreting the CFM mosaics of the tissue fragments, including examining the images at higher magnifications for basic categorization of the tissue into benign and malignant categories and for arriving at a primary histologic diagnosis, was achieved in 3 seconds to 5 minutes. There was no difference in the images generated at the 2 axial depths, including one from the surface and another at a depth of 200 μm beneath the surface of the tissue.

The mosaics of grayscale CFM images of the 55 tissue fragments allowed recognition of the tissue as normal parenchyma of the respective organs in 28 cases (51%), benign tumor in 1 case (2%), and malignant in 26 cases (47%). The grayscale CFM images looked very similar to H&E tissue sections. Focal areas in the mosaic of CFM images showed lack of sharpness of the image, and there were areas that were dark, implying the absence of penetration of light to image focal areas in the tissue. These problems were noted only in small foci in the CFM images. Apart from these problems we did not encounter any other difficulties in the interpretation of the CFM images. Overall, the CFM grayscale images had the required resolution for interpretation and recognition of tissue architecture and cytomorphologic features. The nuclear staining from the acridine orange created the necessary contrast between the nuclear and cytoplasmic areas of the cell, thereby aiding in the recognition of the tissue architecture. The tissue fragments in 28 cases were recognized as normal breast tissue in 8 cases (29%), normal lung tissue in 9 cases (32%), normal kidney tissue in 7 cases (25%), and normal liver tissue in 4 cases (14%). Figure 1 shows representative CFM images, categorized as normal breast, lung, kidney, and liver, with images of the corresponding H&E-stained specimens.

The mosaics of CFM images could be categorized as a benign tumor in 1 case (2%) and as a malignant tumor in 26 of the 55 tissue fragments (47.0%) included in the study. The CFM image of a case of renal oncocytoma resembled the corresponding H&E tissue sections of the imaged tissue. The mosaics of CFM images that were recognized as malignant correlated with conventional histopathologic examination of the H&E tissue sections. Eight breast tissue specimens could be recognized as malignant that correlated with the conventional histopathologic categorization. The breast tissue fragments (n = 8) were recognized as ductal carcinoma in situ in 1 case (12.5%), invasive ductal carcinoma in 6 cases (75%), and invasive lobular carcinoma in 1 case (12.5%), respectively. The overall cytomorphologic features and tissue architecture matched with the corresponding H&E tissue sections.

The 9 tissue fragments obtained from lung resection specimens could be recognized as adenocarcinoma in 7 cases (78%) and as squamous cell carcinoma in 2 cases (22%), based on CFM images that matched with the corresponding H&E tissue sections. Similarly, the CFM images of 6 renal tissue fragments matched with the morphologic findings in the H&E tissue sections and 5 cases of conventional renal cell carcinoma (83%) and 1 case of squamous cell carcinoma (17%). Figure 2 shows the CFM images of a representative case of invasive ductal carcinoma, adenocarcinoma of the lung, and squamous cell carcinoma of the renal pelvis, with images of the corresponding H&E tissue sections. The Table illustrates the details of the tissue fragments that were imaged by CFM technique and compared with conventional histopathologic examination of H&E-stained tissue sections of the imaged tissue.

DISCUSSION

Confocal microscopy allows noninvasive, high-resolution imaging of fresh tissue in its native state.5,17 This optical imaging modality uses the inherent light-scattering properties of the different components of the tissue and can generate optical sections of biologic specimens similar to H&E-stained tissue sections obtained by cutting frozen or fixed tissues. A confocal microscope differs from a conven-
Confocal microscopy can be used for imaging tissues without staining the tissues with fluorescent dyes, which is referred to as confocal reflectance microscopy and CFM. The CFM platform used in our study was easy to use for imaging fresh tissues obtained from our surgical pathology practice. Although there were focal defects in the CFM image, indicating a lack of penetration of light for imaging the tissue, the resolution, architectural details, and overall

Figure 1. Representative confocal fluorescence microscopy (CFM) images of tissue sections of normal breast (A), lung (C), liver (E), and kidney (G). Note that the overall quality of CFM images is good except for focal dark areas. The corresponding hematoxylin-eosin–stained tissue sections are shown in B, D, F, and H (original magnifications ×40 [B] and ×100 [D, F, and H]).
The grayscale CFM images in our study corresponded well with the H&E-stained sections generated from formalin fixation and routine processing of the imaged tissue. We used acridine orange as the fluorescent agent in our study of ex vivo CFM imaging of tissue specimens obtained from surgical excisions. Fluorescent dyes used in ex vivo CFM imaging by other investigators include cresyl violet, indocyanine green, methylene blue, toluidine blue O, and proflavine. These contrast agents result in at least a 1000-fold improvement in nucleus-to-cytoplasm contrast and aid in the recognition of tissues. Proflavine and acridine orange have been used most often for ex vivo CFM. We obtained good results using acridine orange in our study.
specimens were stained optimally and had adequate contrast between the nucleus and cytoplasm, resulting in grayscale images that aided in accurate recognition of the tissue. Similar to other investigators who used acridine orange, we found the integrity of the tissue samples to be maintained in the H&E-stained tissue sections that were prepared following formalin fixation and routine processing of the imaged tissue.

Few previous studies used the same CFM platform that we used in our study to evaluate tissues encountered in surgical pathology practice. Most of these studies explored the utility of CFM for evaluating skin specimens obtained from Mohs's microscopic surgery for the recognition of nonmelanoma cancers, particularly basal cell carcinoma and squamous cell carcinoma. Karen et al27 studied 48 skin excisions, obtained from Mohs surgery, with and without residual basal cell carcinoma of all major subtypes. The overall sensitivity and specificity of detecting residual basal cell carcinoma were 96.6% and 89.2% compared with matched H&E-stained frozen sections of the imaged tissue, providing evidence that CFM can detect residual basal cell carcinoma in Mohs skin excision specimens, with high accuracy. Overall, the studies that used CFM for evaluating skin specimens clearly showed the utility of this noninvasive imaging modality for potential real-time histopathology at the bedside. Dobbs et al27 used CFM to identify neoplasia in breast tissue specimens obtained from surgical excisions. A total of 70 breast tissue specimens from 31 patients were imaged, and 235 regions of interest were compared in detail with corresponding H&E-stained tissue. Neoplasia was identified in histologic images with a sensitivity of 93% and specificity of 97%, compared with 93% for both values with CFM images. Proflavine was used as the fluorescent dye to stain the breast tissues for CFM in this study. In our observations, the authors concluded that CFM can produce images of breast tissue with recognizable architectural features comparable with conventional histology. The same group evaluated the role of CFM for evaluating tumor cellularity in core-needle biopsy specimens obtained from patients with suspected inflammatory breast carcinoma.25 Invasive tumor cellularity estimates from histology and grayscale CFM images showed moderate agreement. The study by Ragazzi et al,34 who tested CFM on different types of surgical specimens, including breast, lymph node, thyroid, and colon tissues, is similar to our feasibility study. Neoplastic tissues were easily distinguished from normal tissue, which is similar to our results. In addition, we are in agreement with them that the use of fluorescence-enhanced contrast and image quality in CFM does not compromise the final histologic evaluation. There have also been a few reports of using CFM successfully for rapid evaluation of brain tissue during neurosurgical procedures.35

The results of our feasibility study indicate that CFM is a promising modality for the rapid examination of tissue fragments of human tissues encountered in routine clinical pathology practice. The occurrence of areas in the mosaics with lack of sharpness of the image and areas that were dark without any image of the tissues are problems that need to be addressed with further improvements in the CFM platform. It is to be noted that we and others have shown the integrity of the imaged tissue to be preserved for subsequent conventional histopathologic examination following formalin fixation and routine processing. However, further studies are warranted to evaluate the integrity of the imaged tissue beyond H&E staining alone to demonstrate optimal preservation for genomic and proteomic analysis. The CFM technique has excellent potential for several applications for tissue qualification in surgical pathology practice if tissue integrity is proven beyond doubt for conventional histopathologic examination and for subsequent ancillary studies.

The CFM technique has several advantages compared with frozen section analysis, which is the most commonly used procedure in surgical pathology practice for rapid examination of fragments of tissue. The procedure of freezing and cutting the frozen tissue block in a cryotome and subsequently staining it for histopathologic examination in the frozen section suite can take up to 20 to 30 minutes. During the freezing process causes artifacts, which can compromise optimal preservation of the tissue specimen. Some tissues, such as those composed mostly of adipose tissue or bone, may cause difficulty during preparation of frozen sections. The CFM technique may be particularly valuable for rapid evaluation of the quality of core needle biopsies, which are generally not subject to frozen section. Optical sections of tissue fragments can be available for viewing using the CFM technique within the acceptable timeline for assessment of the tissue intraoperatively or during interventional radiology procedures. The CFM platform used in our study is suitable for the rapid examination of tissue fragments of varying sizes. Scanning of tissue specimens and image acquisition were accomplished within a few minutes. The quality of the CFM images allowed recognition of the tissue and was comparable to conventional histopathologic examination of the corresponding H&E tissue sections. In addition, it is notable that the CFM technique entailed minimal tissue preparation and did not result in any loss of tissue, unlike frozen section analysis.

### Summary of the Tissue Specimens Evaluated by Confocal Fluorescence Microscopy Technique and Conventional Histopathologic Examination of Hematoxylin-Eosin–Stained Sections

| Tissue of Origin, No. of Cases, and Diagnosis | Breast | Lung | Kidney | Liver |
|---------------------------------------------|--------|------|--------|-------|
| Neoplastic, No. (%)                       | 8 (31) | 9 (35) | 7 (27) | 2 (7) |
| Specific diagnosis, No. (%)               | IDC, 6 (75) | AC, 7 (78) | RC, 1 (14) | AC, 2 (100) |
| Neoplastic, No. (%)                       | ILC, 1 (12.5) | SCC, 2 (22) | SCC, 5 (72) | SCC, 1 (14) |

Abbreviations: AC, adenocarcinoma; DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; RC, renal cell carcinoma; SCC, squamous cell carcinoma.

\( n = 29. \)

\( n = 26. \)
In summary, the relative ease and speed of grayscale image acquisition together with the quality of images that were obtained with the CPM platform used in our study suggest that this technique has promise for use in surgical pathology practice. The CPM images are similar to H&E images, and the use of this CPM technique for possible applications in surgical pathology, such as rapid evaluation of specimen adequacy of core needle biopsy at the time of procurement, margin evaluation of surgical resection specimens, and quality assurance of the tissues for biobanking, needs serious consideration. Our impressive results need further validation with multiple readers in single-institution and multi-institution studies to confirm that the CPM technique can generate images that can allow pathologists to recognize tissues very similar to H&E tissue sections. Such studies together with future investigations to demonstrate tissue integrity for ancillary genomic and proteomic studies will be instrumental in allowing us to realize the potential of this promising technology as a robust, reproducible, and reliable platform for rapid examination of tissue specimens in routine surgical pathology practice.

The authors would like to thank Caliber Imaging and Diagnostic Inc, Rochester, New York, for providing the confocal microscope for the study.

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