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In vivo imaging of ovarian tissue using a novel confocal microlaparoscope

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Abstract

Objectives—Develop a clinical confocal microlaparoscope for imaging ovary epithelium *in vivo* with the long term objective of diagnosing cancer *in vivo*.

Study Design—The first confocal microlaparoscope was developed and used to image the ovaries of twenty-one patients *in vivo* using fluorescein sodium and acridine orange as the fluorescent contrast agents.

Results—The device was tested *in vivo* and demonstrated to be safe and function as designed. Real-time cellular visualization of ovary epithelium was demonstrated.

Conclusions—The confocal microlaparoscope represents a new type of *in vivo* imaging device. With its ability to image cellular details in real time, it has the potential to aid in the early diagnosis of cancer. Initially, the device may be used to locate unusual regions for guided biopsies. In the long term, the device may be able to supplant traditional biopsies and allow the surgeon to identify early stage ovarian cancer.

Keywords

confocal microendoscopy; fluorescence confocal imaging; optical biopsy; ovarian cancer

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Human Experiments: The University of Arizona Human Subjects board approved the protocols used and described in this paper for testing the device on humans. Approval was granted for project number BIO-06-147 on December 22, 2006 and project number 06-0742-04 on October 8, 2008. FDA IND approval number 102603 was obtained to use Acridine Orange *in vivo*.

Conflicts of interest: None.

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Condensation

The first clinical confocal laparoscope capable of performing real-time optical biopsies was developed. The device was used to image the ovaries in 21 patients.

1 Introduction

In this paper, we present the first known use of a confocal microlaparoscope in humans. This new type of laparoscope allows surgeons to obtain nondestructive optical biopsies of tissue in real-time. We present results from a pilot study consisting of twenty-one patients. The device was used to image ovaries *in vivo* prior to laparoscopic oophorectomy.

The American Cancer Society estimates that over 230,000 new cases of ovarian cancer were diagnosed in 2007 while more than 141,000 women died from the disease worldwide.¹ Over eighty percent of all cases are diagnosed during the later stages (Stage III or IV) of the disease when treatment is expensive and generally unsuccessful.² If diagnosis occurs when the cancer is localized to the ovary (Stage I), the survival rate increases to ninety-two percent. The overall prevalence of ovarian cancer is relatively low (1.7 percent lifetime risk). However, the lifetime risk for the disease increases two-fold if the individual has one first or second-degree relative with the disease. With two first-degree relatives the risk increases by twenty-five times (a forty percent lifetime risk).

For the subgroups of women at increased risk, few options exist to allow early detection of the disease. An NIH 1994 consensus stated that there is no single acceptable screening test for ovarian cancer and no evidence that combining the available screening tests—CA125, transvaginal ultrasound, and pelvic exam—has an acceptable sensitivity and specificity.³

Several novel techniques are being developed that could provide clinicians with diagnostic tools to detect early changes in ovarian tissue. Techniques, such as confocal microscopy^{4–12}, two photon microscopy^{14–15}, optical spectroscopy^{16–21}, and optical coherence tomography^{22–28} are able to assess tissue morphology and/or biochemical composition. *In vivo* use of these methods has the potential to provide real-time diagnostic information allowing clinicians to assess the subtle changes that occur early in the disease process. These techniques may increase the sensitivity and specificity of a diagnosis, especially when used as a means for targeting traditional biopsies to regions that can be identified as abnormal at the cellular level.

Confocal microendoscopy, an emerging *in vivo* fluorescence imaging technique, can resolve individual cells and subcellular features. The confocal microendoscope is able to image thin sections of thick samples. This optical sectioning property enables high-resolution microscopic imaging of thick biological samples at moderate depths (typically up to one-hundred to two-hundred microns below the tissue surface).

In vivo confocal imaging has the potential to aid clinicians during screening and/or surgical procedures. Commercial confocal microendoscope systems have recently been developed for visualization of colon and esophagus.⁵ Results have been encouraging in these areas and additional applications for this technology are under investigation. The purpose of this paper is to describe the first clinical confocal microlaparoscope system. In the following sections we describe the new device and present the results of a pilot study.

2 Materials and methods

We have previously reported on the development of a multi-spectral fluorescence confocal microendoscope.^{11,29–32} To evaluate whether such a device could be used to image ovaries *in vivo*, a clinical confocal microlaparoscope system was developed.^{33–37} To create a viable clinical system, several criteria had to be met. First, the microlaparoscope system had to be compact and mobile so that it could be quickly moved into the surgical suite. The microlaparoscope also had to be compatible with standard trocars, be sterilizable, have the ability to deliver small localized volumes of contrast agents, and have the ability to focus at

selected depths. Finally, the device had to be comfortable and easy to use during surgery. A confocal microlaparoscope system was constructed meeting these criteria.

To view a real-time cellular image using the confocal microlaparoscope, the surgeon places the rigid probe through a five millimeter trocar port and contacts the epithelial surface of the organ. Pressing a button on the microlaparoscope handle delivers a controlled volume of fluorescent contrast agent to the field of view. Instantly, a live video of the cells appears on screen. As the probe tip is moved across the surface of the organ, the epithelium can be interrogated in real-time. Pressing another button on the handle saves videos and still frames. By default, the system is configured to image the epithelial layer of cells. However, controls located on the handle allow the surgeon to view deeper cell layers. After use, the microlaparoscope can be disconnected from the system, cleaned, and ETO sterilized for reuse.

In the following paragraphs we describe the confocal microlaparoscope system, discuss the fluorescent contrast agents that were used during imaging, and describe the imaging protocol used to test the device.

2.1 Confocal microlaparoscope system

The confocal microlaparoscope system consists of a microlaparoscope connected to a mobile cart containing a confocal optical scan unit. The microlaparoscope (shown in Figure 1) has a 35 cm rigid probe extending from its handle. The handle contains controls that allow the surgeon to adjust the focus, deliver contrast agents, save still images, and record videos. A flexible cable connects the microlaparoscope to the mobile cart.

The tip of the microlaparoscope's probe contains a miniature 3 mm diameter objective lens that provides a 450 μm field-of-view and a 3 μm lateral resolution. The miniature objective lens images the tissue plane onto a flexible coherent fiber-optic imaging bundle (Sumitomo Electric Industries, White Plains, NY). The fiber bundle runs through a six meter long cable that connects to the optical scan unit, located on the mobile cart. To adjust the imaging depth in the tissue, the focus motor inside the microlaparoscope's handle changes the spacing between the fiber bundle and the objective lens.

To deliver contrast agent to the imaging site, a syringe with fluorescent dye is placed into a spring-loaded port in the handle of the device. The second motor in the microlaparoscope handle acts as a syringe pump that forces the contrast agent through a tiny fluid delivery line in the rigid probe and onto the tissue. The system is able to deliver dye volumes with 50 nL precision.

The mobile cart, shown in Figure 2, contains the optical scan unit, laser, computer, and primary operator console. Live images are visible from the surgical field on a secondary display. The entire mobile unit can be moved into a surgical suite and set up to image within a few minutes.

To collect images, the microlaparoscope illuminates the tissue with laser light. The laser illumination excites the locally delivered contrast agent. Fluorescent signal emitted in the tissue is then collected by the miniature objective lens. The lens images the fluorescent signal onto the distal face of the coherent fiber optic bundle. This signal is then relayed back to the optical scan unit. The optical scan unit is a high-speed confocal microscope. It contains the laser, scan mirrors, optics, confocal aperture, and detector. Detailed technical descriptions of the miniature objective lens, the optical scan unit, and the confocal microlaparoscope system are discussed elsewhere.^{29–32}

2.2 Fluorescent Contrast Agents

Imaging with the microlaparoscope requires the application of an exogenous fluorescent contrast agent. Fluorescein sodium is a dye approved for use in humans for retinal angiography and was selected as the contrast agent for initial testing of the confocal microlaparoscope. The initial clinical study was conducted using approximately 1 to 3 uL of one percent fluorescein sodium per imaging site. This volume is sufficient for staining a 5 mm diameter area on the tissue surface. The localized application of fluorescein sodium also marks the imaging site for correlated biopsies.

Topically applied fluorescein sodium binds rather non-specifically to proteins in tissue. This results in low contrast images that have limited diagnostic value for detecting ovarian cancer. The ovaries were also imaged using acridine orange (AO) following extraction from the patient. AO is a nuclear stain and an excellent fluorophore for visualizing cellular distributions. Its diagnostic potential has been demonstrated previously in the context of detecting ovarian cancer.³⁸

Sites on the ovary that were imaged with fluorescein sodium during the *in vivo* procedure were identified using a UV flashlight. At nearby locations, approximately 1 to 3 uL of 330 uMole/L AO was applied to the surface of the excised ovary and imaged with the microlaparoscope. These sites were biopsied and processed with standard histopathologic procedures using H&E staining.

Towards the end of the pilot study AO was used *in vivo* to validate that the same contrast observed *ex vivo* could be obtained *in vivo*. For *in vivo* testing, 1 uL of 330 uMole/L AO was used to image the ovaries using a protocol that prevented any other organ from coming into contact with the dye. This was accomplished by imaging the ovary inside an endobag. FDA approval to use AO in this context was granted under IND 102603.

2.3 Confocal Imaging Procedure

The pilot study involved twenty-one patients imaged over a one year time period. All procedures in the study were approved by the Institutional Review Board at the University of Arizona. Participants in the study were recruited at the University Medical Center, Tucson, Arizona. Subjects were eligible to participate in the study if they were at least eighteen years of age and not pregnant. All subjects were from the cohort of patients undergoing laparoscopic oophorectomy or open surgery. No financial compensation was offered and there was no diagnostic benefit to the patient for participation in the study.

Standard surgical procedures for clinical oophorectomy were followed with the addition of microlaparoscope imaging prior to ovary removal. Figure 3 shows the microlaparoscope in use during a clinical procedure. All patients were placed under general endotracheal anesthesia and abdominal cavities were insufflated with carbon-dioxide to raise the abdominal wall. The ovary was partially resected and placed inside an endobag to prevent the patient from being exposed to the contrast agent (Figure 4). The microlaparoscope was inserted through a 5 mm trocar. Conventional laparoscopic image guidance was used to position the microlaparoscope tip into contact with the ovary. Several locations on the surface of the ovary were interrogated using the confocal microlaparoscope. In all patients, the extra imaging with the microlaparoscope was completed in under ten minutes.

Once the microlaparoscope imaging was complete, the ovaries were resected, sealed inside the endobag, and removed as part of the standard surgical procedure. Immediately following removal, the ovary was imaged *ex vivo* with the microlaparoscope using AO as the contrast agent. The ovaries were sent to histopathology for clinical diagnosis.

3 Results

Twenty patients were imaged *in vivo* using fluorescein sodium followed by *ex vivo* imaging with AO. One patient was imaged with AO *in vivo*. For the group of patients imaged with fluorescein sodium, seventeen had normal ovaries when evaluated by histopathology and three of the patients were found to have ovarian cancer. For this pilot study the confocal microlaparoscope images were obtained to show clinical feasibility but not used to make a diagnosis. No surgical complications occurred in any of the patients imaged with the microlaparoscope.

In vivo images in Figure 5 and an *in vivo* video in Figure 6 are from ovary stained with fluorescein sodium. As expected, these images have low contrast because fluorescein sodium binds non-specifically to proteins. Much of the observed contrast can be attributed to the distribution of proteins on the tissue surface and uneven pooling of the dye. Although the contrast was limited, we were able to demonstrate that the focus system and dye delivery system performed properly.

Ex vivo images and videos of ovarian tissue stained with AO are shown in Figure 7–Figure 9. These images and videos were collected on *ex vivo* tissue immediately following removal of the ovary. The results show normal ovarian epithelium in Figure 7(a,b) and Figure 8, partially denuded epithelium in Figure 7(c), normal ovarian stroma in Figure 7(d), and adenocarcinoma of the ovary in Figure 7(e–h) and Figure 9. The bright regions in these images correspond to uptake of AO in the cell nuclei. Normal ovarian epithelium typically has an appearance such as that shown in Figure 7(a). The ovary shown in Figure 7(b) has smaller nuclei and microstructural tissue features visible on the surface, which may be associated with a cyst that was observed in pathology. Figure 7(f) and Figure 7(g) were obtained from the same ovary with pathology reporting serous adenocarcinoma and a tumor measuring in excess of 5 mm.

The results suggest that the confocal microlaparoscope has sufficient resolution to differentiate cellular structures and to diagnose cancer. The images show a uniform nuclear distribution for normal tissues and a heterogeneous nuclear distribution for cancerous tissue. The quality of the *ex vivo* images obtained in this study is similar to, if not better than, the image quality observed during another *ex vivo* AO imaging study using a previous version of the confocal microlaparoscope that was able to identify ovarian cancer with a 98 percent sensitivity and 90 percent specificity employing computer-aided diagnosis.³⁸

Figure 10 shows results from the patient imaged *in vivo* using AO. The pathology report diagnosis was serous cystadenoma. The confocal laparoscope's ability to locally identify normal and abnormal regions is highlighted by the two kinds of images obtained in Figure 10. The group of results in Figure 10(a) have the same appearance as normal epithelium as observed *ex vivo* in Figure 7(a). The four images in Figure 10(b) appear abnormal and are believed to have been taken in the region that contained the cystadenoma. The dark region visible in the upper right hand quadrant of the images suggests that the probe may not have been in complete contact with the tissue. Some of the images also appear to show denuding of the epithelium, which may be a result of the partial-resection and placement of the ovary in the endobag before imaging. We expect that with further system optimization and more physician practice using the microlaparoscope, the same quality images seen with AO *ex vivo* will be attainable *in vivo*.

4 Comment

The pilot study showed that the confocal microlaparoscope can be successfully used in the surgical setting for *in vivo* laparoscopic imaging of the ovary. All aspects of the imaging procedure including setup of the instrument, positioning of the microlaparoscope, delivery of

dye, instrument focus, and data collection worked flawlessly without complication. Images obtained *in vivo* with topically applied fluorescein sodium demonstrate that the device functions as designed and that the imaging procedure is safe. Due to the low contrast of topically applied fluorescein sodium, it is uncertain if it can provide sufficient contrast for diagnosis of ovarian pathology. *Ex vivo* images obtained with AO show excellent contrast that highlights cellular morphology. Initial results from *in vivo* use of AO provide preliminary evidence that the same contrast can be obtained *in vivo*.

In the context of this study, AO was used solely to validate that *in vivo* and *ex vivo* contrast was comparable. Although AO has been used clinically on humans in a therapeutic context for the treatment of synovial sarcomas³⁹, its long term safety when used for diagnostic imaging has not yet been established. The dye has not been used extensively in clinical application due to concerns about cytotoxicity and mutagenicity; however acriflavine, a related compound, is currently being used for diagnostic imaging of the colon in Germany.⁴⁰ In terms of optical biopsy, cytotoxicity is not a major concern since tissue extraction biopsy, the current approach, is completely destructive to the interrogated tissue. Mutagenicity, on the other hand, is a concern. We have recently completed a pilot study in our laboratory evaluating the safety of AO and SYTO 16 in a mouse model.⁴¹ The results showed no increase in death rate or cancer incidence in animals treated with a high doses of dye injected into the peritoneal cavity. However, the power of the study was not sufficient to establish safety. Further study is needed before such a dye could be used clinically in a situation where removal of the ovary is not a predetermined outcome.

The development of targeted fluorescent contrast agents with specificity for the molecular signature of ovarian cancer is an active area of research. Combined with the confocal microlaparoscope, these agents may provide the ability to pick up early precancerous changes in the epithelium before the actual onset of disease.

While the confocal microlaparoscope provides excellent *in vivo* images of cells near surface layer of tissue, its imaging depth is ultimately limited to a few hundred microns. It is believed that most ovarian cancers originate in the epithelial layer; however, epithelial cells can line the inner surface of inclusion cysts located well below the surface. Such deep lying structures are not amenable to confocal microlaparoscope imaging, but their presence may be detected by other lower resolution imaging modalities such as ultrasound or optical coherence tomography. The most effective diagnostic tool may be a multi-modality imaging system that combines the capabilities of *in vivo* confocal microscopy with an additional imaging method.

The microlaparoscope represents a new type of *in vivo* diagnostic imaging device. With its ability to image cellular details in real time, it has the potential to aid in the early diagnosis of cancer. Rather than biopsying tissue, sending samples for analysis, and waiting for tissue processing, the microlaparoscope system instantly displays live cellular images at thirty frames per second. Nondestructive optical biopsy enables more extensive interrogation than traditional methods allow. Initially, the device may be used to locate unusual regions for guided biopsies. In the long term, the device may be able to supplant traditional biopsies altogether and allow the surgeon to identify early stage ovarian cancer.

With the success of this pilot study, we will now move forward to quantitatively evaluate the sensitivity and specificity of the microlaparoscope when used to detect ovarian cancer. We will also continue to evaluate potential contrast agents that are safe for *in vivo* use and provide diagnostically useful contrast.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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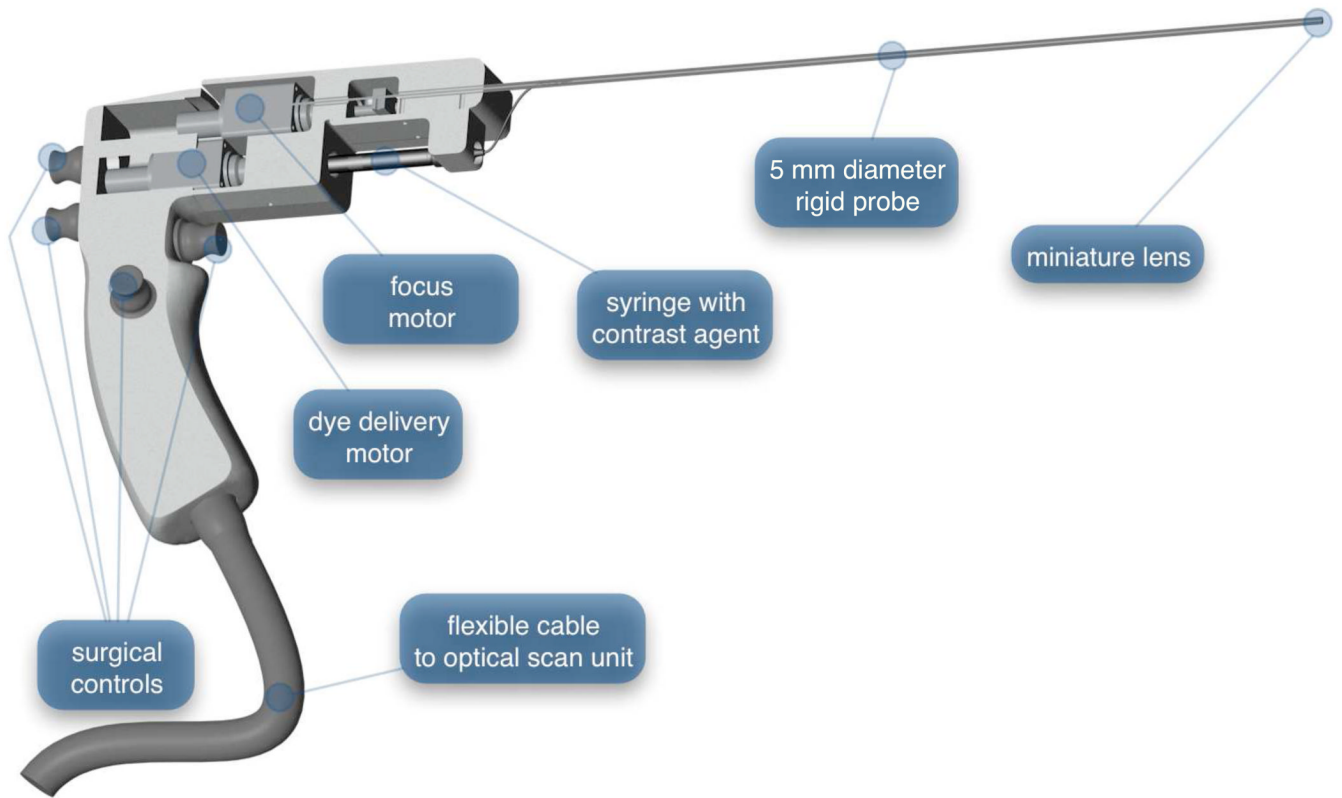


Figure 1. The confocal microlaparoscope. Four push button controls located on the handle allow the surgeon to adjust the focus, deliver contrast agents, save still images, and record videos. Inside the handle, two tiny motors control focus and dye delivery.



Figure 2. The mobile cart. Shelves on the cart, from top to bottom, contain the sterilized microlaparoscope, optical scan unit, laser power supply and system electronics, and computer. The top of the cart holds the operator controls and the primary display.



Figure 3. Confocal microlaparoscope imaging the epithelial surface of an ovary in vivo. The patient's left ovary has been located using a wide field laparoscope (second display from the left). The surgeon on the right (holding the device in his left hand) has inserted the microlaparoscope through a five millimeter trocar port and placed the tip in contact with the ovary. Live cellular images are viewed on the left most display.

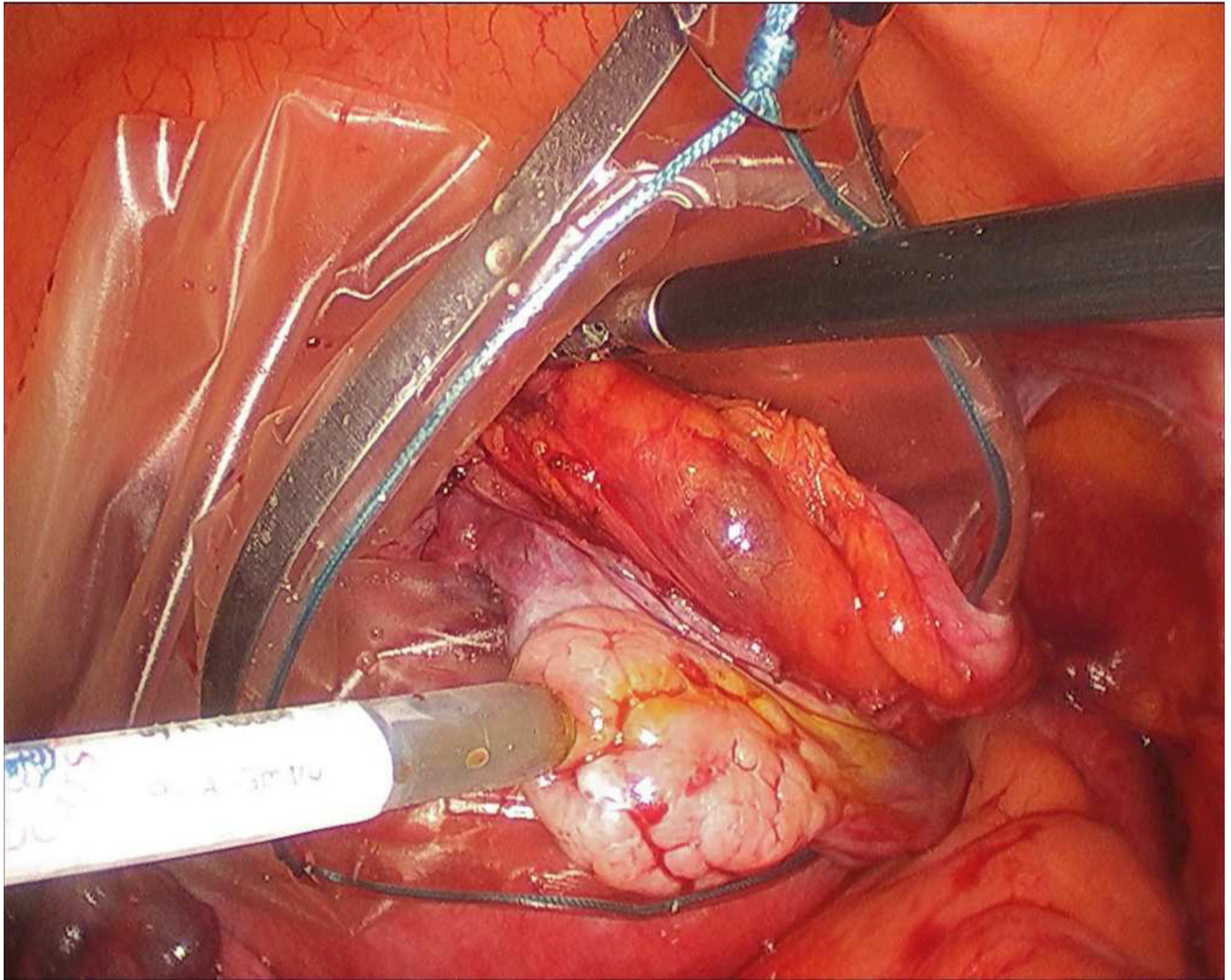


Figure 4.

In vivo imaging of the ovary. Under standard wide field laparoscope visualization, the ovary is located and placed an endobag to prevent other organs from receiving the fluorescent contrast agent. The microlaparoscope (coming in from the lower left) is brought into contact with the ovary, contrast agent is locally applied, and real-time cellular imaging commences.

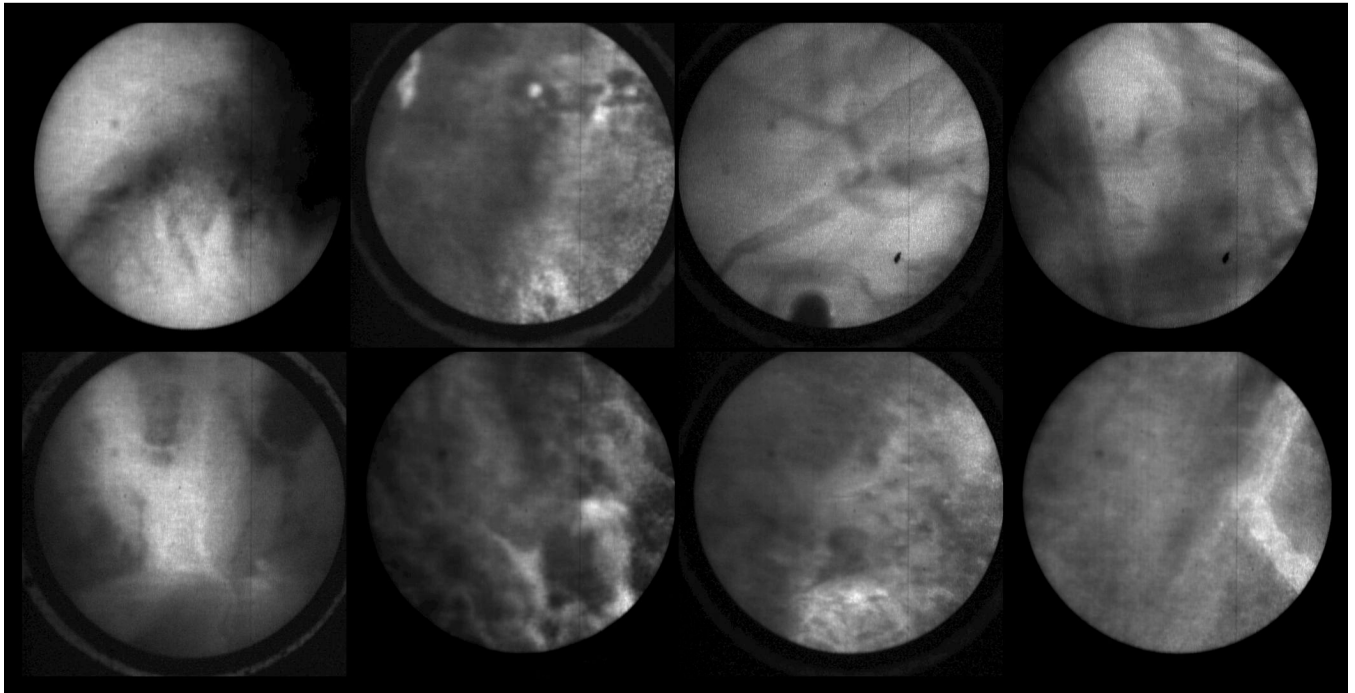


Figure 5.
In vivo confocal microlaparoscope images of the human ovary using topically applied fluorescein sodium as the contrast agent. (Circular field of view is 450 μm .)

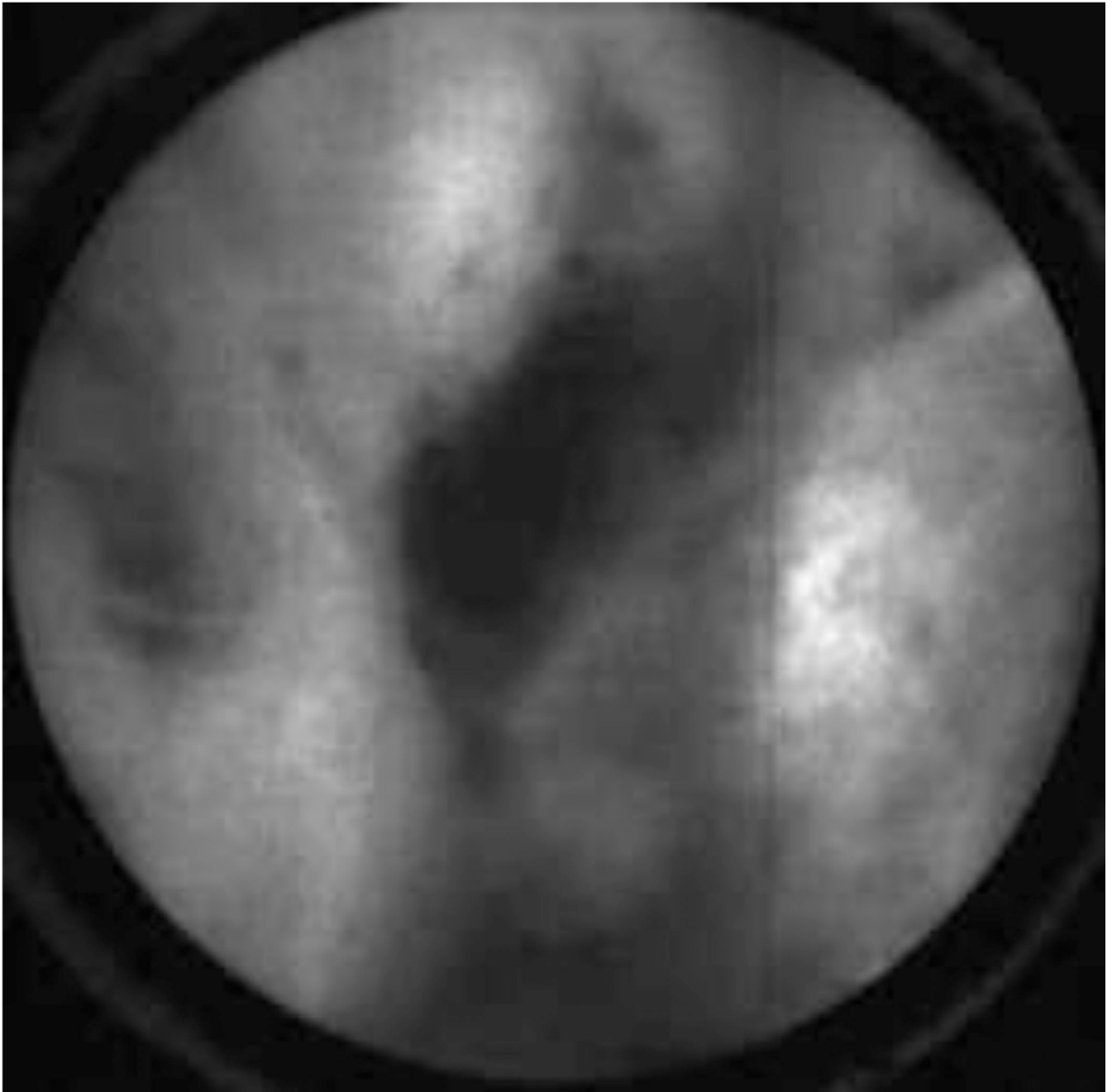


Figure 6. (VIDEO) In vivo confocal microlaparoscope video of the human ovary using topically applied fluorescein sodium as the contrast agent. The pathology report diagnosis was serous cystadenoma. (Circular field of view is 450 μm .)

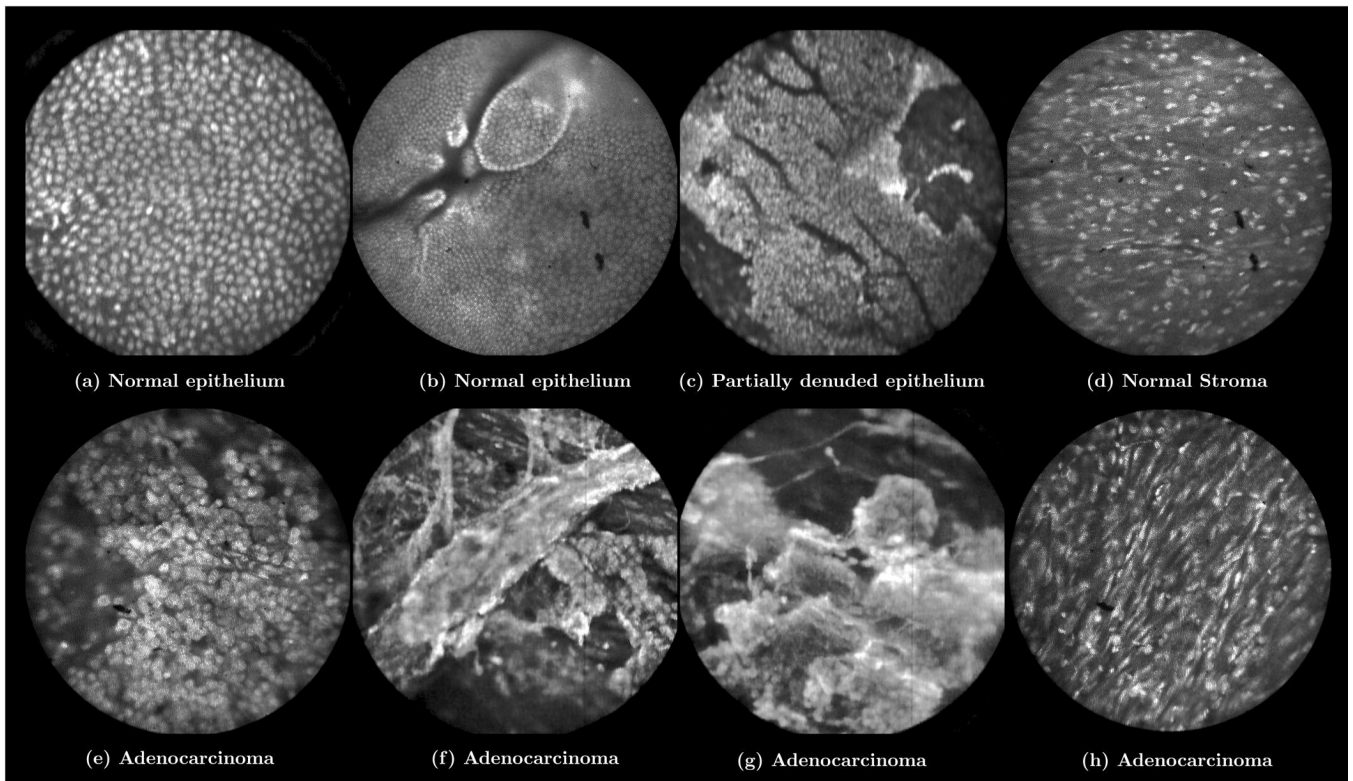


Figure 7. Ex vivo images of ovarian tissue stained with acridine orange. Sub-captions indicate pathology diagnosis. (Circular field of view is 450 um.)

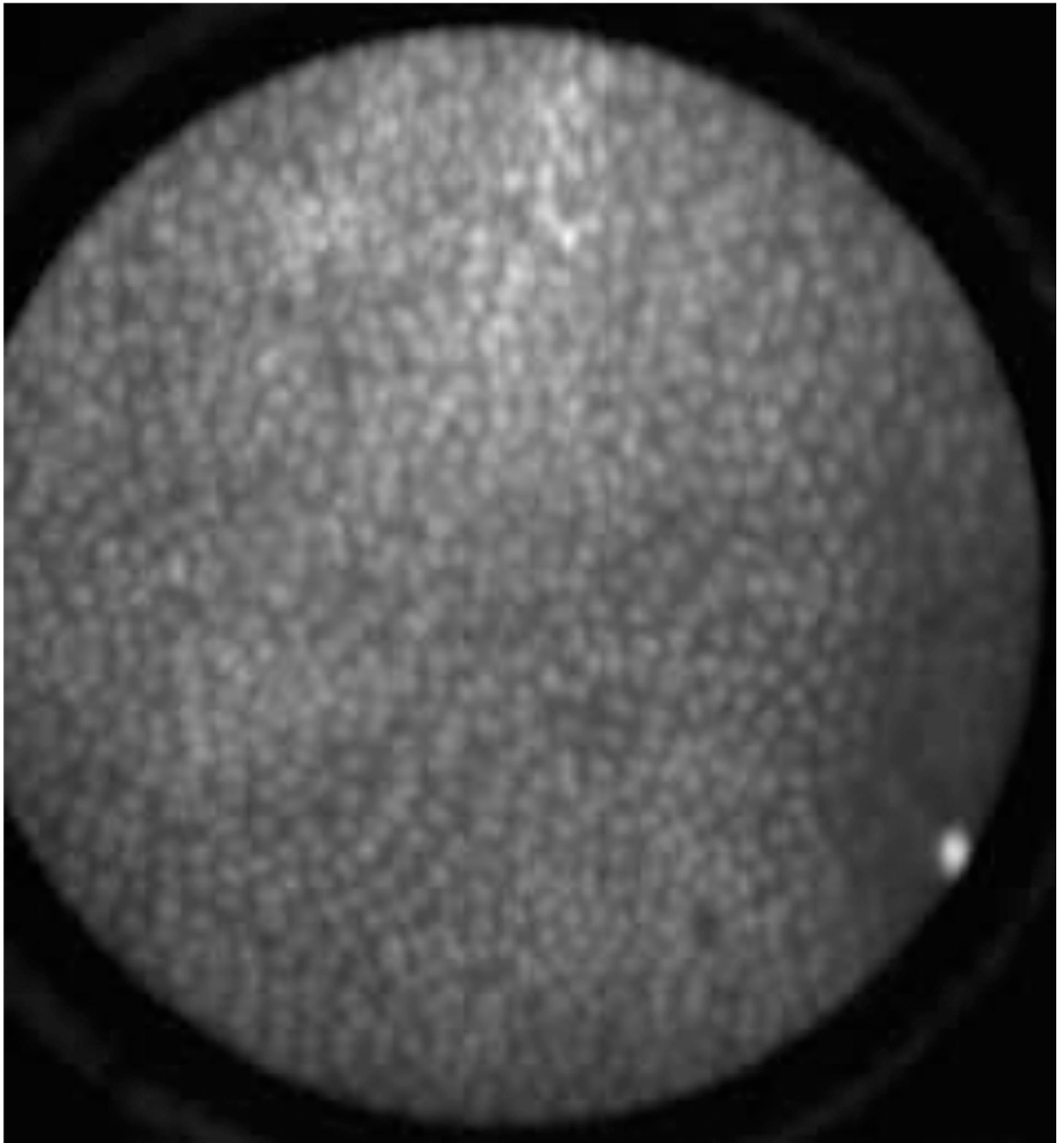


Figure 8.
(VIDEO) Ex vivo video of normal appearing ovary epithelium tissue stained with acridine orange. (Circular field of view is 450 μm .)

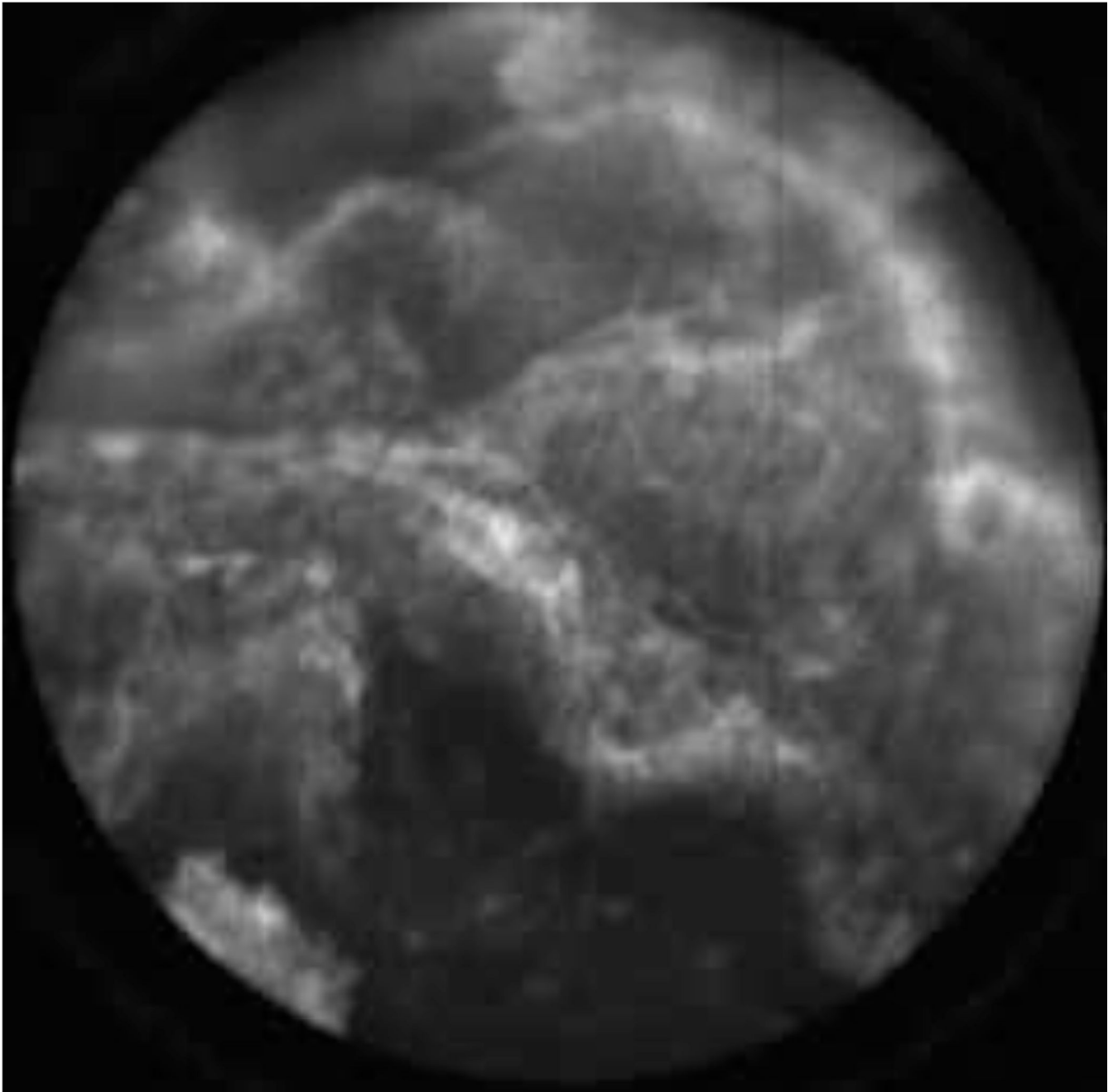


Figure 9.
(VIDEO) Ex vivo video of abnormal appearing ovary epithelium tissue stained with acridine orange. The pathology report diagnosis was serous cystadenoma. (Circular field of view is 450 um.)

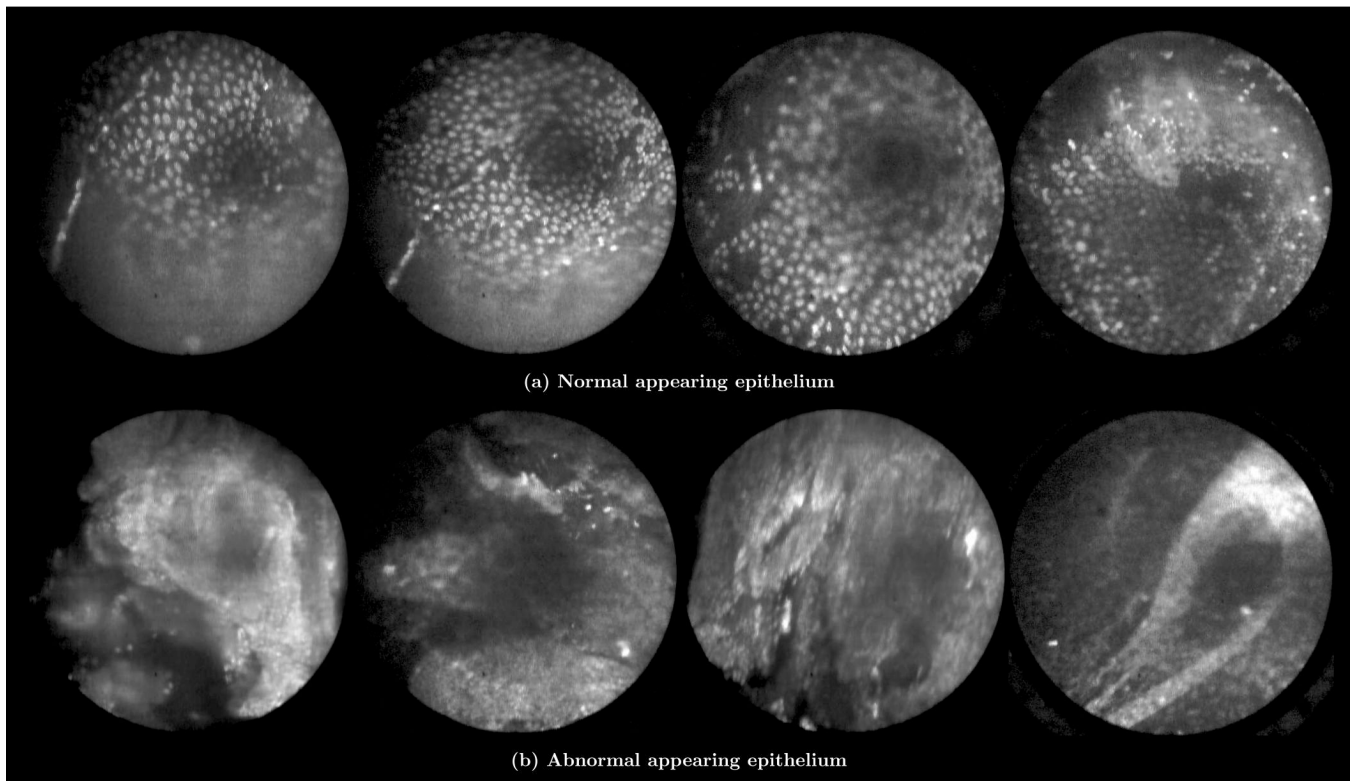


Figure 10.

In vivo images of ovarian tissue stained with acridine orange from the same patient. The top row of images (a) depict epithelial cells that have a normal appearance. The bottom row of images (b) depict epithelial cells that have an abnormal appearance. The pathology report diagnosed the ovaries with serous cystadenoma. (Circular field of view is 450 μm .)