Smartphone epifluorescence microscopy for cellular imaging of fresh tissue in low-resource settings

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Abstract: Disease diagnosis in low-resource settings can be challenging due to the lack of equipment and trained personnel required for histologic analysis. In this paper, we have developed a smartphone-based epifluorescence microscope (SeFM) for imaging fresh tissues at sub-cellular resolution. SeFM provides similar resolution and field of view (FOV) as those used during histologic analysis. The SeFM device achieved the lateral resolution of 0.57 µm and provided microscopy images over a sample area larger than 500 µm. The material cost was low, approximately $3,000. Preliminary images of human pancreatic tumor specimens clearly visualized cellular details. Quantitative analysis showed that using an excess dose of a chemotherapy drug significantly reduced the tumor-specific fluorescence signal, confirming the specificity of the drug and the detection potential of SeFM.

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1. Introduction

Microscopic examination of excised tissues has been playing a critical role in modern medicine. During the diagnoses of many diseases, the suspicious lesion is excised from the patient and prepared as a histology slide. The pathologist then uses a microscope to examine the slide for disease-associated cellular changes. While microscopes are becoming more affordable, the resources and trained personnel required for preparing the excised tissue into a histologic slide are still scarce in low- and middle- income countries (LMICs). Therefore, disease diagnosis is often made without the histologic analysis, which can lead to inaccurate or delayed diagnoses [1] and consequently inadequate treatment.

Recently, several low-cost, smartphone-based microscopy devices have been developed to improve disease diagnosis in low-resource settings [2–10]. Most of these smartphone microscopes, however, are designed to image thinly-sectioned tissue slides and might have limitations in low-resource settings without the pathology lab. For many of the smartphone microscopes reported, a singlet (e.g., half-ball lens, aspheric singlet) is used as the objective lens. The singlet approach provides an economical solution for achieving high resolution at the center of the image but its useable field of view (FOV) is limited by the aberrations. As a result, simultaneously achieving high resolution and sufficient FOV as used during histologic analysis can be challenging. Even at the center of the field, the resolution reported with singlet-based
smartphone microscopes is limited to 1-10 µm [11], which might not be sufficient to visualize certain sub-cellular details examined during the high-power field microscopy with the resolution of ~ 0.5 µm. The smartphone can be directly mounted on an existing bench microscope to utilize well-corrected objective lenses [12]. In this approach, the microscope-to-smartphone interface needs to be carefully designed to avoid vignetting and fully utilize the resolution and FOV that the objective lens can afford. In addition, use of the existing tube lens in the bench microscope might make it challenging to build a compact, portable smartphone microscope.

Freshly-excised tissues can be directly imaged with microscopy without the resource-intensive slide preparation process. Fluorescent dyes such as acridine orange or proflavine can be topically applied on the tissue to provide fluorescence signals from cell nuclei [13–15]. Other tissue contrasts such as scattered signal [14] or second-harmonic generation (SHG) signal [13] can be used to visualize the morphology of the surrounding cellular structures. Microscopic imaging of the fresh tissue can be conducted by two-photon microscopy [13], confocal microscopy [14,15], or light-sheet microscopy [16]. The images obtained with these microscopes provide comparable histomorphologic accuracy to the hematoxylin and eosin (H&E)-stained slide images that are commonly used during histologic analysis. Fresh tissue microscopy also has been shown to provide high diagnostic sensitivity and specificity for breast [13,15] and skin cancers [17]. Recently, UV surface excitation was used to image fluorescence signals from fresh tissues [18], which can provide a low-cost microscopy solution for imaging fresh tissues. While the aforementioned works have separately demonstrated the feasibilities of constructing smartphone-based microscopes and visualizing cellular features from freshly-excised tissues, a smartphone-based microscope optimized for imaging fresh tissues with a similar performance to bench microscopes has not been reported yet.

In this paper, we report the development of a smartphone-based, epi-fluorescence microscope (SeFM) that is tailored to image fresh tissues in low-resource settings. In our SeFM device, we carefully designed the microscopy optics to utilize the high resolution and full FOV that the objective lens can provide and to ultimately generate images that are comparable to the histologic slide images. In addition, we tested imaging of fresh tissues stained with a tumor-specific fluorescent dye, PARPi-FL [19,20], which can provide high diagnostic accuracy for the target disease and facilitate rapid, quantitative analysis.

2. Methods

2.1. Smartphone epifluorescence microscope (SeFM)

Schematic of the SeFM device is shown in Fig. 1. In the illumination path (side view, Fig. 1), excitation light from the LED (central wavelength = 470 nm; bandwidth = 22 nm) was refracted by the condenser lens (plano-convex singlet; f = 50 mm). The excitation light was further transmitted through the short-pass excitation filter (edge wavelength = 492 nm), reflected by the dichroic mirror (edge wavelength = 500 nm), and finally focused by the objective lens (CFI Plan Achromat 40x, Nikon; f = 5 mm; NA = 0.65) onto the sample. The focal length of the condenser lens and distances between the LED, condenser lens, and objective lens were determined using an optical simulation software, Optics Studio (Zemax, WA), to provide uniform illumination over the target FOV of 500 µm (RMS intensity variation: 4% of the average intensity) without any observable LED emission area pattern while achieving useable light throughput (31%).

In the detection path (front view, Fig. 1), emitted light from the sample was captured and collimated by the objective lens. The emission light was then transmitted through the dichroic mirror and long-pass emission filter (edge wavelength = 501 nm). The camera lens (f = 25 mm; f-number = 0.95) generated an intermediate image of the sample with the magnification of 5. The intermediate image was relayed to the CMOS imaging sensor inside the smartphone (iPhone SE, Apple) using two smartphone camera lenses, one inside the smartphone and the other right in front of the smartphone camera. The excitation filter, dichroic mirror, and emission filter were
chosen to image fresh tissues stained with PARPi-FL, a fluorescent dye developed for imaging nuclear enzyme Poly(ADP-ribose)Polymerase 1 (PARP1). PARP1 is known to be upregulated in a multitude of different cancers but not in adult normal tissues. PARPi-FL has previously been shown to provide high sensitivity and specificity for detecting oral cancer [21]. The same filter combination can be also used for imaging with acridine orange to visualize cell nuclei.

2.2. Microscopy optics design

In order to fully utilize the maximum NA and full FOV that the objective lens can provide, we optimally designed the relay optics. Our target FOV was 500 µm and target resolution 0.5 µm, similar to the FOV and resolution used during histologic analysis. This resulted in 1,000 resolvable points (\(= 500 / 0.5\)) for one lateral axis. More than 2,000 pixels needed to be used per lateral axis to achieve the Nyquist sampling. With the pixel size of the smartphone CMOS sensor of 1.19 µm, the image needed to be mapped on a sensor area larger than 2,380 µm (\(= 2,000 \times 1.19\) µm). This determined the minimum value for the overall magnification between the sample plane and CMOS sensor, 4.76 (\(= 2,380 / 500\)). When a relay optics is used between the objective lens and smartphone camera (Fig. 2), the overall magnification is determined by the following equation:

\[
M_{\text{overall}} = \frac{f_{\text{smartphone}}}{f_{\text{objective}}} M_{\text{relay}},
\]

where \(f_{\text{smartphone}}\) is the focal length of the smartphone camera lens, \(f_{\text{objective}}\) is the focal length of the objective lens, and \(M_{\text{relay}}\) is the magnification of the relay optics.

With the given focal lengths of the objective lens and smartphone camera lens (5 mm and 4.15 mm, respectively), the minimum required magnification of the relay optics was determined as 5.73 (\(= 4.76 / 4.15 \times 5\)). This minimum magnification can be achieved by numerous combinations of two readily available lenses. In order to reduce the size and cost of the device, we chose to use an additional smartphone camera lens (iPhone SE rear camera lens; \(f = 4.15\) mm; f-number = 2.2) as the second lens of the relay optics. The smartphone camera lens is ideal for this purpose due to its low cost (<$10), good optical performance over large FOV (several mm), and relative high NA (~0.2) [5]. This makes the minimum focal length of the first lens of the relay optics as 23.8 mm (\(= 4.15\) mm \(\times 5.73\)). We have used a large-aperture, multi-element camera lens (Speedmaster, Mitakon Zhongyi; \(f = 25\) mm; f-number = 0.95) as the first lens of the relay optics. The final magnification between the sample and CMOS sensor was 5.

After the two lenses in the relay optics were determined, we calculated the optimal distance between the objective lens and camera lens to ensure that the fluorescence light from the entire target FOV of 500 µm was coupled to the CMOS sensor without any vignetting. We
first measured the locations of the focal planes and pupils and the pupil diameters of each optical element relative to its mechanical outer housing. An imaging setup composed of a CMOS sensor (acA800-510um, Basler) and CCTV lenses with various focal lengths was used in conjunction with a micrometer-driven translation stage to measure these key locations and pupil diameters. Figure 2 shows two conditions that needed to be met to ensure no vignetting through the microscopy optics. In both conditions, rays originating from the edge of the FOV (half field size = 0.25 mm) were investigated. Pupils are represented as two vertical lines with the central opening. In the first condition (Fig. 2(a)), the marginal ray that passes through the bottom edge of the exit pupil of the objective lens (marginal ray 1) needs to enter the smartphone camera lens entrance pupil below its top edge (red dotted circle in the inset, Fig. 2(a)). The objective-to-camera lens distance that satisfied this condition was 89 mm. In the second condition (Fig. 2(b)), the marginal ray that passes through the top edge of the exit pupil of the objective lens (marginal ray 2) needs to enter the camera lens entrance pupil below its top edge (red dotted circle in Fig. 2(b)). The distance between the objective lens and camera lens that satisfied this condition was 137 mm. When designing the custom 3D-printed optics holders of the microscope, we set the distance between 89 and 137 mm.

2.3. Performance testing

The lateral resolution was tested by imaging a USAF resolution target and a Ronchi grating (50 lines/mm). Both USAF resolution target and Ronchi grating were made of a glass substrate with the chrome pattern. Since the reflection of the excitation light by the chrome pattern was mostly rejected by the emission filter, a sheet of white paper was placed on the back of the glass substrate to generate the green fluorescent light, which was used as the back illumination to reveal the fine chrome features as shadows. The full-width-half-maximum (FWHM) of the line-spread function (LSF) was calculated. Fluorescence imaging capability was tested by imaging a lens cleaning paper and an H&E-stained swine esophagus histology slide.

Fresh tissue imaging capability was tested by imaging human pancreatic tumor specimens stained with PARPi-FL. The human tissue imaging protocol was approved by the Memorial Sloan Kettering Cancer Center Internal Review Board. Two specimens were imaged using PARPi-FL alone (100 nM) or a mixture of 100 times excess dose Olaparib and PARPi-FL as a negative control. PARPi-FL was diluted in 30% PEG/PBS and samples were incubated for 5 minutes before being quickly washed in PBS. Olaparib is a chemotherapy drug that target-inhibits PARP1.
and was used in this experiment to exclude the possibility of false-positives in the PARPi-FL fluorescence imaging. The two pancreatic tumor samples were then imaged using the SeFM device. The images were acquired by the default Camera application of iPhone SE. The exposure time and ISO value were automatically adjusted by the Camera app to 1/17 and 2,000, respectively. Images were saved in the JPEG format. SeFM images were clearly visible without any noticeable background noise under typical indoor lighting conditions. A piece of black paper or plastic was placed on the backside of the sample to further reduce coupling of the ambient light into the SeFM device. From our previous studies of imaging with PARPi-FL, we found that PARPi-FL is stable at room temperature and does not lose its optical properties under typical indoor lighting conditions, which further enabled the SeFM imaging in a standard lab or office setting.

Image analysis and processing were conducted using ImageJ [22]. The histogram-equalized image was generated by converting the original image into an RGB stack, equalizing the intensity histogram within each color channel, and re-converting to an RGB color image. Both SeFM images without and with Olaparib were histogram-equalized based on the intensity histogram of the image without Olaparib. The autofluorescence-reduced image was generated by converting the original image into an RGB stack, multiplying the pixel intensities of the red channel by 1.5 times, subtracting the multiplied red-channel image from the green-channel image, creating a new RGB stack with the (green – red × 1.5) image into the green channel and a zero-intensity image as the red and blue channels, and converting the RGB stack into an RGB color image. The fluorescence signal amount was measured for the autofluorescence-reduced images. For each image, four distinctive 125 µm × 125 µm regions of interest (ROIs) were identified and the average intensity for each ROI was calculated. The average and standard deviation of the four intensity measurements were reported for each autofluorescence-reduced image.

3. Results

A photo of the SeFM device is shown in Fig. 3(a). The device was 21 cm × 16 cm × 20 cm and weighed 3.4 kg. The material cost was approximately $3,000. The list of the components along with the price information is provided in Table 1. Maximum illumination power on the sample was 15.4 mW. The illumination power was adjustable with a potentiometer connected to the LED driver. A representative image of the USAF resolution target is shown in Fig. 3(b). The magnified view (inset, Fig. 3(b)) shows that the smallest line pattern at group 9, element 3

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Fig. 3. Photo of the SeFM device (A) and representative image of the USAF resolution target.
(line width = 0.78 µm) is clearly resolved. The measured FWHM of the LSF was 0.57 ± 0.10 µm. The measured FWHM was comparable to the resolution used during the high-power field microscopy. The image size corresponded to 725 µm × 544 µm on the sample plane. A circular region with a diameter of approximately 648 µm contained signals from the specimen. The RMS intensity variation within the 648 µm diameter was 21% of the average intensity. The intensity variation within the target FOV of 500 µm was 7%. The measured FOV was similar to a typical high-power field size used during histologic analysis, 450 µm × 450 µm.

| Component                              | Price  | Component                | Price  |
|----------------------------------------|--------|--------------------------|--------|
| **Optical components**                 |        | **Electrical components**|        |
| Objective lens                         | $406   | iPhone SE                | $140   |
| Camera lens                            | $319   | LED                      | $25    |
| iPhone SE lens                         | $18    | LED mount                | $36    |
| Dichroic mirror                        | $335   | LED socket               | $4     |
| Emission filter                        | $305   | Heat sink                | $77    |
| Excitation filter                      | $355   | Battery (12V)            | $24    |
| Fold mirrors (×2)                      | $191   | LED current drive        | $20    |
| Condenser lens                         | $33    | Power switch             | $4     |
| **Mechanical components**              |        | **Potentiometer**        | $14    |
| Sample stage                           | $22    |                          |        |
| Focusing tube                          | $80    |                          |        |
| Objective lens adapters                | $39    |                          |        |
| 3D printing materials (181 in³)        | $456   | **Total**                | $2,903 |

A fluorescence microscopy image of a lens cleaning paper is shown in Fig. 4(a). The detailed structure of the fibers is clearly visualized. A representative image of an H&E-stained swine esophagus histology slide is shown in Fig. 4(b). The left side of the image exhibits collagen fibers in lamina propria and the right side squamous epithelium. In the magnified view (inset, Fig. 4(b)), squamous epithelial cells are well visualized with the dark cell nuclei and brighter cytoplasm.

Images of the human pancreatic tumor specimen stained with PARPi-FL are shown in Fig. 5. The RGB image in Fig. 5 is presented in its original JPEG format without any post-processing.
such as image filtering or brightness/contrast adjustment. Numerous tumor cells are shown with clear subnuclear details such as nucleoli (inset). Linear structure (arrows) is also present in this image, showing collagen autofluorescence typical of tumor masses. While the tumor cells and collagen appear to have similar color in the original RGB color image, only the collagen exhibits strong signals in the red channel. Green and blue channels show both tumor cells and collagen. This suggests that the fluorescence signal from the collagen is red-shifted and has a broader bandwidth compared to the specific fluorescence signal from the tumor cells [19]. The image quality achieved with the SeFM was comparable to those obtained with the standard fluorescence microscope and confocal microscope [23].

Figure 6 shows the images of the PARPi-FL-stained pancreatic tumor specimens without and with an excess dose of Olaparib. The original images show that the intact specimen (Fig. 6a) exhibits significantly stronger fluorescence signals compared to the specimen co-incubated with Olaparib (Fig. 6d). Histogram-equalized images (Figs. 6b, e) visualize the tumor cells and collagen (arrows) with perceivably different colors, which might be helpful in analyzing molecular-specific details (visualized by PARPi-FL fluorescence) in reference to the tissue intrinsic structures (visualized by autofluorescence). The histogram-equalized images (Fig. 6b, e) also provide better overall image quality than original images (Fig. 6a, d) due to the use of wider ranges of color and brightness. Finally, autofluorescence-reduced images (Figs. 6c, f) show a low signal from the collagen (arrows). Quantitative analysis showed that the fluorescence intensity in the autofluorescence-reduced image is significantly higher in the intact specimen than in the Olaparib-co-incubated specimen, 12.4 ± 1.1 vs. 3.9 ± 0.7.
4. Discussion

In this paper, we have developed a SeFM device and demonstrated microscopic imaging of fresh human tissues. The preliminary results show that SeFM can visualize tumor-specific cellular details along with intrinsic tissue structures. With the ongoing development of rapid-staining, molecular-specific fluorescent dyes for fresh tissues, we believe SeFM will enable the microscopic examination of the freshly-excised human tissues in low-resource settings and facilitate the timely and accurate diagnosis. The SeFM device will be readily utilizable in outpatient clinics in LMICs, where patients from rural areas can travel to and receive diagnosis and treatment during a single visit. Due to the presence of autofluorescence in certain tissue types, wide-field smartphone fluorescence imaging with low resolution might face challenges in accurately detecting malignant tissues. With the high resolution and image quality comparable to the standard histology microscopy, we expect that the SeFM will provide an affordable and accurate diagnostic solution.

While our first SeFM prototype was constructed with the material cost of $3,000, we expect a significant reduction of the device cost for commercially-viable SeFM devices by procuring components in large quantity and mass-producing mechanical holders through standard manufacturing methods such as injection molding rather than 3D printing. With the reduced device cost and further development of automated image analysis tools, we expect that the SeFM device can be directly deployed in remote areas and used by low-cadre healthcare workers. The tumor-specific fluorescence dye used in this paper, PARPi-FL, is a small molecule-based imaging agent with a long shelf life and is expected to be available at a low cost in the near future.

We have found several technological areas that need improvement. Different tissues likely have different RGB color ratio for the autofluorescence, which might pose challenges in reducing the autofluorescence signal during quantitative analysis. For the given clinical application of examining a particular tissue type, many specimens from different patients can be imaged to
measure the variation and average of the RGB color ratio of the tissue autofluorescence. If the variation is found small, the average value might be useable for a pre-determined color channel subtraction method. An adaptive color ratio measurement method based on histomorphologic features might need to be developed for more general tissues. The tissue might not be flat to the cover glass, which will make some portion of the image out of focus [16]. To mitigate this issue, multiple images can be obtained at different imaging depth levels generating a composite image with a sharp focus over the entire FOV. In order to ensure that all the suspicious regions are examined and to minimize the operator’s sampling mistake or bias, an automated method of translating the specimen might be needed to image the entire specimen area.

In addition to the aforementioned improvements, future SeFM devices can be modified to provide flexible fluorescence imaging capabilities and utilize ever-advancing smartphone technologies. The SeFM optics was constructed with multi-element lenses that are well-corrected for chromatic aberrations and therefore will be useable for other excitation and emission wavelengths. Future SeFM devices can be modified to incorporate exchangeable filter sets and multiple LEDs with different colors for imaging various fluorophores. While the iPhone SE smartphone was used in the current SeFM device because of its affordable cost at the time of development ($140) and sufficient number of pixels to conduct Nyquist sampling of the microscopy image, other smartphones can be readily integrated into the SeFM device by modifying the mechanical holder design and using an adequate external smartphone camera lens. We expect that newer smartphones will provide better image quality with improved sensitivity and reduced sensor noise and facilitate image processing and analysis with enhanced computing power.

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WZ and DK are the inventors of the provisional patent application based on the SeFM technology presented in this paper. CB and TR are shareholders of Summit Biomedical Imaging, LLC. TR is a co-inventor on a filed U.S. patent (WO2016164771) that covers methods of use for PARPi-FL. TR is a co-inventor on a U.S. patent (WO2012074840), covering the composition of matter for PARPi-FL. TR is a paid consultant for Theragnostics, Inc.

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