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Efficient Confocal Microscopy with a Dual-Wedge Scanner

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ABSTRACT
Confocal microscopes achieve high spatial resolution by focusing both a light source and a detector to a single point with an objective having a high numerical aperture. In order to produce an image, it is then necessary to scan this common focal spot through the specimen, and it is often important to image the full field of view in a short time. In order to avoid vignetting, the scanning must be done in or near the pupil of the optical system. For some fast scanners, this requires the use of multiple relay telescopes to create multiple pupils. Galvanometric scanners impose a practical limit on the scan speed because of the angular accelerations involved in reversing direction. Rotating polygons are often used to achieve greater speed. For a scanner consisting of a rotating polygon and a galvanometric scanner, two relay telescopes are normally used.

We have developed a dual-wedge scanner which has the potential to perform the scan in a configuration which is very short in the axial direction, thereby eliminating much of the complexity of current high-speed scanners. We have demonstrated a prototype of the scanner in a reflectance confocal microscope. Transverse and axial resolution are comparable to those of other scanning systems.

The selection of rotation speeds for the wedges is important to ensuring full coverage of the field of view in a reasonable time. Various tradeoffs on these parameters will be discussed. The beam behavior in the pupil will be discussed. Resolution limits and aberrations will be shown from ray-tracing analysis, and compared to experimental results.

1. INTRODUCTION
There are 1.2 million new cases of basal–cell and squamous–cell skin cancer (BCC and SCC) diagnosed every year in the US alone, and these non–melanoma skin cancers are increasing in number every year.1, 2 Clinical screening and diagnosis include some 5.5 million biopsies and histologies performed every year, of which 80% turn out to be benign and might have been avoided, had better non-invasive screening techniques been in clinical use. Considering that the cost of a biopsy and histology is approximately $500, the 4.4 million benign biopsies cost US health-care $2.2 billion and in addition left patients with pain, scarring, and mental anguish that could have been avoided had better imaging capabilities been available for in vivo diagnosis. Confocal reflectance microscopy may enable such non–invasive clinical screening and diagnosis, in addition to pre–surgical determination of the cancer margin, and intra–surgical guidance directly on the patient in real-time, all without the need for biopsy.

Confocal reflectance microscopes have demonstrated excellent success in imaging nuclear and cellular morphology of human skin in vivo.3–11 These microscopes have been cleared by the FDA for use to assist in skin cancer diagnosis and have led to more than 70 papers by dermatologists at leading academic medical centers in the USA and Europe. Thus far, confocal reflectance imaging has demonstrated significant promise for clinical applications such as guiding skin biopsy, determining the margins of skin cancers to guide surgical excision, non-invasive monitoring of the efficacy of laser and topical drug treatments, and sensitivity and specificity studies of screening and diagnosis of skin cancer,8, 12–34 Depth of imaging is limited to a few hundreds of micrometers, but most skin cancers originate in the epidermis or near the dermal–epidermal junction which is 50 to 150μm
deep and easily amenable to imaging with confocal reflectance microscopy. However, the beam scanning mechanism makes these microscopes large and expensive, hindering clinicians from using them on a regular day-to-day basis to image more lesions, including those which currently are difficult to reach. A small, robust, hand-held, inexpensive, and user-friendly microscope would make such imaging much easier and more common, and could expand confocal microscopy into other areas of the body.

2. DUAL–WEDGE SCANNING

The use of two prisms to scan a laser beam was first described by Rosell in 1960 as a prism scanner. Since then, the scanner has been utilized in various applications under a variety of names that include rotating prism scanner for optical tracking in guidance systems, dual-wedge scanner for laser radar, Risley prism scanner, double-prism scanner for testing the performance of inter-satellite laser communications, and a paired-angle rotation scanner for an OCT scanning probe.

2.1. Scanning Concept

The concept of the scanner is shown in Figure 1. A prism with wedge angle $\alpha$ is oriented such that the second surface is normal to the incident, collimated laser source and with an angular position $\theta_1$ about the optical axis, where the angular position is measured from the apex of the prism. The incident laser beam is deviated by the vector $\vec{v}_1$ in the sample plane in Figure 1(a). As the prism is rotated about the optical axis ($\theta_1 = 0$ to $2\pi$), the beam scans the dotted circle. When an identical prism is placed in the path such that the angular positions of the prisms are the same ($\theta_1 = \theta_2$) and the flat surfaces are parallel and facing each other, the beam will be deviated by the vector sum $\vec{v}_1 + \vec{v}_2$ in Figure 1(b). If the first prism is held stationary at $\theta_1 = 0$, and the second prism was rotated about the optical axis.
second prism is rotated about the optical axis \((\theta_2 = 0 \text{ to } 2\pi)\), a circle with radius \(|\vec{v}_2|\) will be scanned about the endpoint of \(\vec{v}_1\) in Figure 1(c). When the prisms are rotated in the same direction and at the same speed \((\omega_1 = \omega_2 = \omega)\) over time \(t\), the angular positions of the first and second prisms are \(\theta_1 = \omega t\) and \(\theta_2 = \omega t + \Delta\theta\), respectively, where \(\Delta\theta\) is the angular offset between the two prism apexes, and the beam scans a circle with a radius dependent on \(\Delta\theta\). If the prisms rotate in the same direction, but at different speeds \((\omega_1 \neq \omega_2)\), the angular relationship between the two apexes will change with time and a spiral scan will be produced. When the prisms are rotated in opposite directions and at the same speed, the angular positions of the prisms are \(\theta_1 = \omega t\) and \(\theta_2 = -\omega t + \Delta\theta\), respectively, and the beam scans a line at an angle \(\Delta\theta\). If the prisms rotate in opposite directions, but at different speeds, the angular relationship between the two apexes will change with time and a rosette scan will be produced. Various scan patterns are generated by changing the relative rotation speeds between the prisms and/or the angles of the prisms.\(^{36,38}\) However, it is important to note that using prisms with different wedge angles will miss a circular region with a radius \(||\vec{v}_1| - |\vec{v}_2||\) within the center of the scan. Our work involves prisms with equal apex angles.

The analysis is slightly more complicated than it would seem, because the effect of the second prism is slightly dependent on the angle at which light emerges from the first. An exact ray tracing analysis of the prism rotations is used to calculate the final refraction vector.\(^{36,37}\)

### 2.2. Prototype Dual–Wedge Microscope

A benchtop microscope\(^{41}\) which incorporates the dual–wedge scanner, is shown in Figure 2. The optical path is similar to that of most confocal microscopes except for the scanning technology. The path begins at the 830–nm, linearly–polarized laser diode, and passes through the polarizing beamsplitter with \(P\)–polarization. The \(P\)–polarized light continues through the dual–wedge scanner. For experimental purposes, we also include a relay telescope that places the scanner (\(P1\) in the figure) conjugate to the pupil of the microscope objective (\(P2\)), and allows flexibility in changing objectives. The scanner houses two 11–degree prisms \((\alpha = 11^\circ)\) within two aluminum cylinders, each within a pair of ball bearings and a timing belt pulley. Two timing belts couple identical pulleys on the shafts of the motors and the prism housings to ensure a one–to–one ratio between the prism and the motor rotations. The beam passes through a quarter–wave plate, having an axis oriented at 45° relative to the linear laser polarization, thereby producing circularly polarized light, which is then focused into the sample by the custom–built, infinity–corrected, water-immersion 30X objective, with a numerical aperture of 0.9. Water–immersion provides better correction for aberrations introduced by skin and reduces specular reflection. The infinity–corrected lens has been specifically designed to image tissue by including a basic tissue model in the optimization process to minimize aberrations. The tissue model includes multiple surfaces representing index matching fluid between the disposable window and the stratum corneum and the multiple layers of tissue. Each layer is assigned optical properties and a thickness. These properties are varied over ranges expected in human skin, and optimized using a figure of merit which includes the multiple configurations. Optimizing the design for multiple configurations has yielded a design that has acceptable performance across varying imaging conditions for different tissue depths and refractive indices within human skin. Unlike standard objective lenses, that generally are designed to image through either a thin glass cover–slip or none at all, the custom–built objective was designed to image through a thick (0.762 mm, 0.030inch) polycarbonate window. The thick window is mounted within a ring that is placed directly on the skin to reduce axial motion and keeps the tissue stable while imaging a patient with motion caused by respiration and heartbeat.

The backscattered light that is collected within the numerical aperture of the objective passes back through the quarter–wave plate, telescope, and scanner. On the return path, the light has passed through the quarter–wave plate a second time converting the incident \(P\) polarization into \(S\), provided the backscattered light retains its state of polarization as it interacts with the sample. Light that has not retained its polarization has likely been scattered multiple times, and the fact that some of this light is rejected by this technique improves contrast in optical sections. The \(S\)–polarized light is reflected by the polarizing beamsplitter toward the avalanche photodiode (APD), and is focused by a lens through a pinhole onto the detector.

Data streams are acquired from the two encoders and the APD module. A National–Instruments NI USB–6251 High–Speed, USB M–Series, multi–functional data acquisition card samples the two encoders that are
attached to the motors and the voltage output of the APD (i.e. backscattered light that is detected) simultaneously. A look-up table is used in conjunction with the angular positions to place the intensity value in the correct pixel location. The look-up table is constructed by modeling the orientation of the prisms and calculating the refraction vector through each of the four surfaces. Resulting \((x, y)\) coordinates are truncated to the nearest pixel location. A dot of brightness proportional to the signal amplitude from the APD, digitized in 12 bits, is placed at the appropriate location on the display.

3. OPTICAL PERFORMANCE

Figure 4 shows the current axial response function, for two different pinhole sizes, as determined by moving a mirror axially through the focus of the objective. Hair gel is used as an index–matching material between the objective and the mirror to mimic the refractive index of water in a horizontal configuration. The width of the axial response function with the 50\(\mu m\) pinhole is 1.8\(\mu m\), which approaches the expected value\(^{42}\) of

\[
\delta z = 0.95 \frac{n \lambda}{NA^2} = 0.95 \frac{1.33 \times 830nm}{0.9^2} = 1.3\mu m.
\]

The 100\(\mu m\) pinhole was used to collect images, and the measured FWHM is 2.1\(\mu m\).

After the resolution was determined, we collected an image of excised human skin that is normally discarded after Mohs surgery, shown in Figure 5. The pixel size is set at 2\(\mu m\), to acquire a good image with the slower scan speed of the benchtop instrument. The skin was sandwiched between a microscope slide and a cover–slip with the epidermis toward the cover–slip, and the cover–slip was coupled to the immersion objective with hair gel. The image shown, is at a depth of approximately 35\(\mu m\), and demonstrates the optical sectioning and ability to resolve features such as hair follicles and cells.

4. SCAN PATTERN ANALYSIS

Current confocal microscopes produce a raster scan with about 500 pixels by 500 lines, and the goals for the dual–wedge scanner is to achieve the same number of scan points in 1/5 second, with full coverage of a circular field of view and minimal repetition of points. For analysis of the pattern, it is sufficient to use an approximation:

\[
x = (n – 1)\alpha (\cos 2\pi f_1 t + \cos 2\pi f_2 t) \tag{2}
\]
\[
y = (n – 1)\alpha (\sin 2\pi f_1 t + \sin 2\pi f_2 t), \tag{3}
\]
Figure 4. AXIAL RESPONSE FUNCTION: The width of the axial response function decreases with decreasing pinhole size, and is near the expected value.

For the location of the focused spot. The diameter of the field of view in the image plane is thus twice the maximum of either $x$ or $y$, or

$$D_F = 4(n - 1)\alpha. \quad (4)$$

The diameter in the object plane is this value divided by the magnification of the system. Taking derivatives of Equations 2 and 3 with respect to $t$, it can be shown that the distance between data points a time $\delta t$ apart is

$$\delta r = \sqrt{\delta x^2 + \delta y^2} = 2\pi \delta t (n - 1)\alpha \sqrt{f_1^2 + f_2^2 + 2f_1 f_2 \cos [2\pi (f_1 - f_2)t]}, \quad (5)$$

with extreme values of

$$2\pi \delta t (n - 1)\alpha |f_1 + f_2| \quad \text{and} \quad 2\pi \delta t (n - 1)\alpha |f_1 - f_2|. \quad (6)$$

The pixel time, $\delta t$, is the time between samples. To obtain approximately one pixel between consecutive points, based on our 500²-pixel grid, we want,

$$\delta r \approx \frac{(n - 1)\alpha}{500\pi}, \quad (7)$$

or

$$2\pi \delta t (n - 1)\alpha |f_1 + f_2| \approx \frac{(n - 1)\alpha}{500\pi} \quad (8)$$

$$\delta t \approx \frac{1}{2 \times 500\pi^2 (|f_1| + |f_2|)}. \quad (9)$$

For rotations speeds in the thousands of Hertz, the ideal pixel time will be less than a microsecond. If the ratio of rotation speeds, $f_2/f_1$ is a rational number, then the pattern will be repeated. Specifically, if the ratio is an irreducible fraction,

$$\left| \frac{f_2}{f_1} \right| = \frac{N_N}{N_D}. \quad (10)$$

Figure 5. SAMPLE IMAGE: An image of excised human skin was obtained using the new configuration. The diameter of the field of view is 1.0 mm.
the number of lobes in the rosette scan will be
\[ \ell = N_N + N_D, \quad (11) \]
and the larger this number, the more dense the data will be. For the 500^2 grid, the ideal value is
\[ \ell = N_N + N_D \approx 500\pi. \quad (12) \]
The pattern will then repeat after a time
\[ t_{\text{rep}} = \frac{N_D}{f_1} = \frac{N_N}{f_2}, \quad (13) \]
after which no new information will be obtained.

We have examined scans with a variety of possible speed ratios that determine the fill factor, or the fraction of all points in the 500^2 fixed grid that are visited at least once in the frame time. We found a very complicated behavior, a small part of which is shown in Figure 6. If the number of lobes in Equation 11 is small, the fill factor becomes very low, and fewer pixels will be sampled. Exploring the whole range of \(-f_1 < f_2 < f_1\), the fill factor is relatively constant at a value dependent on the pixel time and frame time. Figure 7 shows the fill factor as a function of the frame time. We see that it reaches half its maximum value at 1/5 second. Figure 8 shows the fill factor as a function of the pixel frequency, \(f_p\). The fill factor approaches its maximal limit at 1 MHz, after which additional increase in frequency results in multiple visits to each pixel. The figure also shows the number of pixels visited at least twice, and at least five times.

As an analytical demonstration, we use a speed ratio \(f_2/f_1 = -331/1399\), with the first wedge rotating at 50,000 RPM, which produces a pattern having 1730 lobes, that repeats every 1.7 seconds. Figure 9 shows the sampling for three different frame times. The left panel shows that we will obtain a coarse image in 1/20 second with the center of the image having noticeably better resolution. The center panel shows the sampling at the nominal frame time of 1/5 second. Even better resolution can be obtained in multiple frame times, but the improvement diminishes asymptotically as is evident in Figure 7.

This figure suggests a number of ways of using the scan data effectively. After only 1/20 second, a high density of data is available in the center of the image, while a coarse image is obtained in the peripheral region. This is suggestive of the foveal vision of the human eye, and could be used for a quick analysis to identify a region of interest. If this data is used to produce an image, the user can search large areas, and pause when an area of interest is found. Pausing at any location, the image resolution in the periphery would improve, producing an image of nearly the same resolution as the raster scan after 1/5 second. Further dwelling on the same region would lead to slightly improved resolution, and continuous improvement in signal-to-noise ratio.
With any digitized image, the data must be sampled. In many confocal microscopes, the sampling is regular, using a raster scan. It is important to compare the scan patterns generated using the dual–wedge scanner with those of a raster scan, to determine the extent to which it is possible to reproduce all the relevant spatial frequencies in an image. The ideal raster scanned image is sampled at least at the Nyquist frequency of a band–limited image, and then according to the sampling theorem, all the signal information can be recovered. The limitation on spatial bandwidth is normally the aperture of the objective. In practice, images are often under-sampled according to this criterion. Satisfactory results are obtained because the image has little energy at high spatial frequencies and because, in confocal microscopy, the image is partially band–limited by the size of the pinhole in front of the detector.

In the present implementation of the dual–wedge scanner, data points are assigned to the nearest pixels that are located in a pre–defined raster pattern. At completion of the imaging process, any pixels that were not visited by the scanner are filled with the data from the nearest visited pixel. The processing is simple, but does not make the best use of the data. Analysis of the scan pattern can be used to develop optimal scan patterns given that the image is band–limited in spatial frequency by the numerical aperture of the objective.

Better means for interpolation of data points will lead to better images, and the ultimate capability is limited only by fundamental principles of sampling. Just as the Nyquist theorem describes the conditions under which perfect reconstruction is possible using regularly sampled data, there are other fundamental limitations relating to irregularly spaced data points. To address these issues, several portions of the 1/5 second image in Figure 9, were expanded, and examined in detail. One is shown in Figure 10(a). For this example, a 30X objective was assumed, with a field of view of 1.17 mm. The Fourier transform of the sampling function is shown in Figure 10(b). For comparison, a raster scan is shown in Figure 10(c,d). The regular sampling in the raster scan (10c) produces a symmetric pattern in spatial frequency (10d). The bright points are at the inverse of the sampling frequency, and the remaining points are the result of the finite window around the region of interest. The sampling rate is 500 pixels per mm.

The sample of the dual–wedge scan shows similar results, with the spacing of sample points in adjacent lines (10b) of about 1000 cycles per mm. This spacing is about twice as good as the 500–pixel–square images that are frequently used in confocal microscopy. Variations in line spacing occur (10a) because the pattern has not completely filled in at this time. The pattern in spatial frequency (10b) is a distorted version of the one for the raster scan in Figure 10(d). It is interesting to note that very high resolution can be obtained for objects which are large in a direction perpendicular to one of the scan lines. Thus, with appropriate signal processing,
for images which are band–limited in spatial frequency, it should be possible to produce an image equivalent to that obtained with the raster scan.

6. SUMMARY

Confocal reflectance microscopy has been demonstrated to be useful for imaging skin lesions. A dual–wedge scanner can result in an extremely compact microscope with the potential to image in areas which are less accessible, and to do so at a reduced cost.

In summary, the dual–wedge scanner can be implemented with achievable scan speeds, and will produce a high–resolution image without missing pixels provided that scan frequencies are chosen appropriately. Image quality may be improved by signal processing to make maximal use of the spatial frequencies being imaged at each point in the scan.

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