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Modulated-alignment dual-axis (MAD) confocal microscopy optimized for speed and contrast

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Abstract

Modulated-alignment dual-axis (MAD) confocal microscopy combines the benefits of dual-axis confocal (DAC) microscopy and focal-modulation microscopy (FMM) for rejecting out-of-focus and multiply scattered light in tissues. The DAC architecture, which utilizes off-axis and separated beam paths for illumination and detection, has previously been shown to be superior to single-axis confocal (SAC) microscopy for the spatial filtering (rejection) of unwanted background light. With the MAD approach, a modulation of the alignment between the illumination and collection beam paths tags ballistic photons emanating from the focal volume with a characteristic radio frequency that can be extracted and separated from background signal using lock-in detection. We report here an optimized form of MAD confocal microscopy where we have fully mitigated tradeoffs in performance in an initial proof-of-concept system in order to recover the imaging speed of DAC microscopy while retaining contrast enhancement of 6 dB (signal-to-background ratio) with a secondary improvement in optical-sectioning and inplane resolution. Validation is demonstrated with light-scattering tissue phantoms and freshly excised tissues.

Index Terms

biomedical optical imaging; optical microscopy; optical modulation; amplitude modulation; acousto-optical devices; fluorescence; optical signal detection; optical design

I. Introduction

The tradeoff between imaging resolution and contrast (which affects imaging depth) is widely recognized in the field of biomedical optics [1], constraining many modalities such

as optical coherence tomography [2], two-photon microscopy [3], structured-illumination microscopy [4], diffuse optical tomography [5] and photoacoustic tomography [6]. Many of these imaging modalities have been applied towards clinical needs such as point-of-care optical biopsy for both outpatient diagnostic screening as well as intraoperative guidance [7]. Within the realm of microscopy, several approaches have been explored in recent years to improve tissue-imaging performance, including illumination techniques to enhance light delivery to the microscope's focal volume [8], detection techniques to distinguish in-focus light from background light [9] or specimen manipulation techniques to enhance transparency to light or otherwise minimize unwanted perturbations that inevitably arise in light-tissue interactions [10].

For microscopy applications where the depth of imaging in optically thick specimens is of primary concern, several techniques achieve high contrast (signal-to-background ratio) by operating either in regimes where there is minimal background signal (e.g., two-photon microscopy [11] and SRS microscopy [12]) or in regimes where in-focus signals have very different characteristics than the background (e.g., focal-modulation microscopy (FMM) [13], spatial overlap modulation microscopy [14], optical lock-in detection [15] and ultrasound-encoding [9], [16]), thereby providing a means of filtering out the background signal to boost the ability of these systems to image deeply into biological specimens.

Dual-axis confocal (DAC) microscopy (Fig. 1) is an approach that achieves superior contrast and imaging depth by spatially separating the illumination and collection paths, which results in stronger rejection of out-of-focus and multiply scattered light than conventional single-axis implementations of confocal microscopy that employ a common path for tissue illumination and the collection of light [17]-[23]. For example, Monte-Carlo simulations of DAC and SAC microscopes were performed, as described previously [18], in which the FWHM optical-sectioning thickness (axial resolution) of the microscopes were matched. The axial response to a plane mirror (Fig. 1(c)) translated through the focal plane (defocused) away from the focus demonstrates superior rejection of out-of-focus and multiply scattered light in DAC compared with SAC. Simulations were performed for various optical lengths (OL) of scattering, in which the OL is defined as the total number of scattering mean free paths traveling by ballistic photons along a perpendicular round-trip path from the tissue surface to the mirror and back out of the tissue. Similarly, a simulation of a reflective knife edge translating laterally through the focus of the microscopes demonstrates superior performance in DAC compared with SAC (Fig. 1(d)). In all cases, it is clear that the DAC configuration provides enhanced contrast (signal to background ratio, SBR) compared to the SAC microscope configuration.

In modulated-alignment dual-axis (MAD) confocal microscopy, we intend to preserve imaging resolution while providing an additional mechanism to enhance contrast (signal-to-background ratio, SBR). This is implemented by introducing a modulation of the spatial alignment between the illumination and collection paths of a DAC microscope. Spatial modulation on the order of several microns generates a strong amplitude modulation of the focal volume signal with minimal amplitude modulation of the background. Since this radio-frequency signature is unique to the focal volume, it can be separated from the relatively unmodulated background through lock-in detection. The first proof-of-concept prototype

of a MAD confocal microscope [24], however, gained this additional imaging contrast by sacrificing imaging speed (five times slower) to accommodate hardware limitations involved with spatial modulation.

In this paper, we report advances in MAD confocal microscopy that allow for the recovery of the imaging speed of previous DAC microscopy systems while maintaining MAD microscopy's superior contrast enhancement. In addition, axial and in-plane resolution have been improved. We delineate various modifications that were made to optical components and instrumentation and examine their effects on axial and transverse contrast (SBR) in an idealized tissue phantom and on imaging performance in freshly excised fluorescently labeled mouse tissues.

II. Methods

A. Optical System Design

The optical circuit (Fig. 2(a) and Fig. 3(a)) of a MAD confocal microscope system, from laser source to image generation, is as follows. A 660-nm laser source (OBIS FP 660LX, Coherent, Santa Clara, CA) is coupled to a single-mode fiber (SMF, NA = 0.11), collimated (L₁, 1.45-mm EFL [C140TMD-B], ThorLabs, Newton, NJ) and directed into the entrance aperture of an acousto-optical deflector (1250C-BS-960A, Isomet Corporation, Springfield, VA). After exiting the acousto-optical deflector (AOD), the zero-order and diffracted-order collimated beams are refocused (L₂, 3.1-mm EFL [C330TMD-B], ThorLabs, Newton, NJ) towards a slit (S50R, ThorLabs, Newton, NJ) that only allows the 1st-order diffracted light to continue through the system. The 1st-order diffracted beam that passes through the slit is re-collimated (L_3 , 25.0-mm EFL [NT49-660], Edmund Optics) and subsequently refocused (L₄, 18.4-mm EFL [KGA280-B-MT], Newport Corporation, Irvine, CA) to the focal volume of the microscope through an index-matching hemispherical solid-immersion lens [21] (QU-HS-6, ISP Optics, Irvington, NY). Light from the focal volume is collected by a set of lenses that are identical to the illumination optics (L_3 and L_4) but offset at an angle (2θ) of 60 deg with respect to the illumination optical axis. The collection optics couple light from the focal volume into a single-mode fiber, which acts as a pinhole to remove out-of-focus and multiply scattered light. Collected light is converted to a voltage signal using a PMT detector (H7422-40, Hamamatsu, Edison, NJ) in conjunction with a transimpedance amplifier (DHPCA-100, FEMTO, Berlin, Germany). The voltage signal is then read by a spectrum analyzer (FSEA20, Rohde&Schwarz, Munich, Germany) operating as a lock-in amplifier. The spectrum analyzer generates a voltage output (video signal) that corresponds to the strength of the modulated signal. This video signal is digitized (PCI-6115, National Instruments Corporation, Austin, TX) and displayed as pixel intensities by a custom framegrabber written in LabVIEW (National Instruments, Austin, TX). Rasterscanned imaging (vertical sectioning) is achieved (Fig. 3(b)) using a galvanometric scan mirror (6210H Series, Cambridge Technology, Bedford, MA) to scan the illumination and collection beams within the sample in the horizontal (y) direction (fast axis) while a piezoelectric actuator is used to scan the sample in the vertical (z) direction (slow axis) (P-601.4SL, Physik Instrumente LP, Auburn, MA). For volumetric microscopy, a motorized actuator scans the sample in the third dimension (x direction) (LTA-HL, Newport

Corporation, Irvine, CA). The pixel dwell time is maintained at 625 ns for both DAC and MAD modes (PCLK = 1.6 MHz).

B. Principle of Operation

Given that the focal volume of a DAC microscope is determined by the superposition of two symmetric optical paths that focus to and intersect at the same point (Fig. 1(b) and Fig. 2(a)), MAD confocal microscopy exploits the dependency of the system's optical throughput on the alignment between these two optical paths to tag focal-volume signals with a characteristic temporal frequency. In MAD confocal microscopy, the AOD sinusoidally scans the position of the focus in the illumination path (at the focal volume of the microscope) relative to the fixed position of the collection beam by a certain amplitude (y/ω_0). By carefully optimizing the maximal alignment offset (modulation depth) between these two optical paths (Fig. 2(b) and Media 1), expressed as the ratio between the distance separating the paths to the beam waist size y/ω_0 , the optical signal from the focal volume can be efficiently tagged with a characteristic frequency that is much less present in the background signal, thereby allowing for efficient isolation of the in-focus signal using lock-in detection. For an alignment offset modulation rate of f, the illumination focus passes through the focal volume (collection focus) twice per cycle, generating amplitude modulation of the in-focus signal at a frequency 2f(Fig. 2(c)).

Regarding the lock-in amplifier's ability to isolate the frequency of interest, it is important to consider the number of signal cycles that will be integrated. Obtaining the narrowest pass band possible, centered at 2f, would require integrating over an infinite number of 2f cycles, which would yield a delta-function pass frequency [25]. At the other extreme where only a small number of 2f cycles can be integrated in order to realize a reasonable imaging speed (Figure 4), the lock-in amplifier's transfer function amplitude, expressed as follows, provides the effective filter bandwidth [26]:

$$|H(f)| = \frac{2}{\pi N} \frac{\sin(N\pi f/f_0)}{1 - (f/f_0)^2}$$

where N is the number of 2f cycles that is integrated by the lock-in amplifier and $\frac{f}{f_0}$

is the normalized frequency. The original MAD system generated pixel intensities after integrating over 6.25 cycles of 2f signal (2f= 1.6 MHz), yielding an effective lock-in amplifier bandwidth (-3 dB) of 483 kHz while the increased imaging speed of the optimized system reported here integrates over 4 cycles of 2f signal (2f= 6.4 MHz) with negligible effect on performance, yielding an effective lock-in amplifier bandwidth (-3 dB) of 650 kHz (see Results and Discussion).

III. Results and Discussion

Due to the finite acoustic velocity within the AOD crystal, the maximum frequency at which the AOD can spatially modulate the alignment of the illumination beam scales inversely with input beam diameter. This, in turn, limits the imaging speed because the intensity

of each image pixel is determined by the number of periods of 2f signal that must be integrated by the lock-in amplifier before another pixel can be imaged. As a result, the original MAD confocal microscopy system was constrained to image at a frame rate that was five times slower (0.4 Hz vs. 2.0 Hz) than our standard DAC microscope systems (assuming an equivalent field of view). In the optimized MAD system, the input beam diameter ($1/e^2$) was reduced from 0.99 mm to 0.32 mm to allow faster AOD modulation rates (0.8 MHz vs. 3.2 MHz). However, the finite acoustic velocity in the AOD's crystal implies that excessively high modulation rates will achieve smaller and smaller angular scan ranges at a given driving voltage, which was mitigated partially by driving the AOD's internal voltage-controller oscillator with larger input voltage amplitudes.

Another important limiting factor of the original AOD (1205C-2, Isomet Corporation, Springfield, VA) used in the first proof-of-concept system [24] was that the optical power of the 1st-order output beam varied as a function of alignment offset, which introduced a weak but appreciable amplitude modulation in the illumination beam itself. This, in turn, generated a background signal that competed with focal-volume signal modulation at exactly the same lock-in frequency (2*f*). Therefore, the optimized MAD system uses an acoustic beam-steered AOD (1250C-BS-960A, Isomet Corporation, Springfield, VA), which employs two acoustic transducers phased in such a way as to maintain a more constant optical power in the first-order light as a function of alignment offset (acoustic driving frequency centered at 150 MHz).

Experimental examination of the relationship between the normalized contrast (SBR) from a mirror target in water, as a function of modulation depth (y/ω_0) and modulation frequency (Fig. 5), demonstrates that the optimal modulation frequency increases initially to a global maximum in both the original and newly optimized MAD system before descending as frequency increases. The optimal driving frequency is much higher for the new MAD system (~1.8 MHz) compared to the original [24] slower MAD system (~0.5 MHz). The optimal modulation frequency is increased for the new system reported here since, as mentioned previously, the beam diameter of the illumination beam entering the AOD has been reduced, which enables a faster response to the varying acoustic frequencies within the AOD.

Axial and transverse performance were measured to ensure that the described modifications, which were implemented to improve the MAD system's speed, did not inadvertently lead to a sacrifice in resolution or contrast. Experimental results (Fig. 6) demonstrated that contrast enhancement was preserved in a 20% Intralipid (Fresenius Kabi, Uppsala, Sweden) scattering phantom at levels of 5-6 dB axially (improved SBR for MAD vs. DAC). Additionally, improved axial and transverse resolution was achieved by shortening the effective focal length of the specimen-side lenses, L_4 , from 25.0 mm to 18.4 mm to increase the NA of the beams. The initial MAD system provided optical-sectioning (axial) resolution of $2.9-3.0 \mu m$ (FWHM) while our optimized MAD microscope has a resolution of $2.6-2.7 \mu m$. In-plane resolution was also improved from $1.9-2.0 \mu m$ to $1.7-1.8 \mu m$ (FWHM).

In tissue-imaging experiments, freshly excised kidney and colon tissues from euthanized mice were stained for five minutes at 4-deg C using AlexaFluor 660 (S21377 [lot #1366517], Life Technologies Corporation, Carlsbad, CA) at a concentration of 10 mg/mL

and rinsed for one minute in PBS before imaging. With an illumination power of 0.7 - 0.9 mW at 660 nm, and an imaging speed of 2 frames per second (pixel rate of 1.6 MHz), volumetric datasets were recorded, from which *en face* images could be rendered (Fig. 7). In photon-limited conditions when imaging deeply in tissues, the PMT provides excellent sensitivity. While this superior sensitivity is in exchange for limited dynamic range, the lock-in amplification of the MAD signal helps to recover the weak modulation signal buried within the noise floor (future work will investigate other detectors such as avalanche photodiodes for their potential advantages with the MAD microscopy technique). The images reveal an enhancement in image contrast that is consistent with what was observed in our Intralipid scattering-phantom measurements (Fig. 6). Collectively, these data demonstrate the ability of MAD confocal microscopy to improve imaging contrast (SBR) in turbid media at penetration depths of approximately $100 \, \mu m$.

IV. Conclusion

MAD confocal microscopy operates best at intermediate depths where there is sufficient scattering to be rejected but not so much scattering that there is insufficient in-focus signal to be extracted (a photon-starved condition). In comparison to an initial MAD prototype, the optimized MAD confocal microscope described here achieves imaging rates of 2 frames per second (1.6-MHz pixel sampling rate), which is equivalent to DAC microscopy systems reported previously [21]. In addition, spatial resolution has been improved with the optimized MAD system while maintaining strong contrast enhancement of 6 dB (SBR) in comparison to DAC microscopy.

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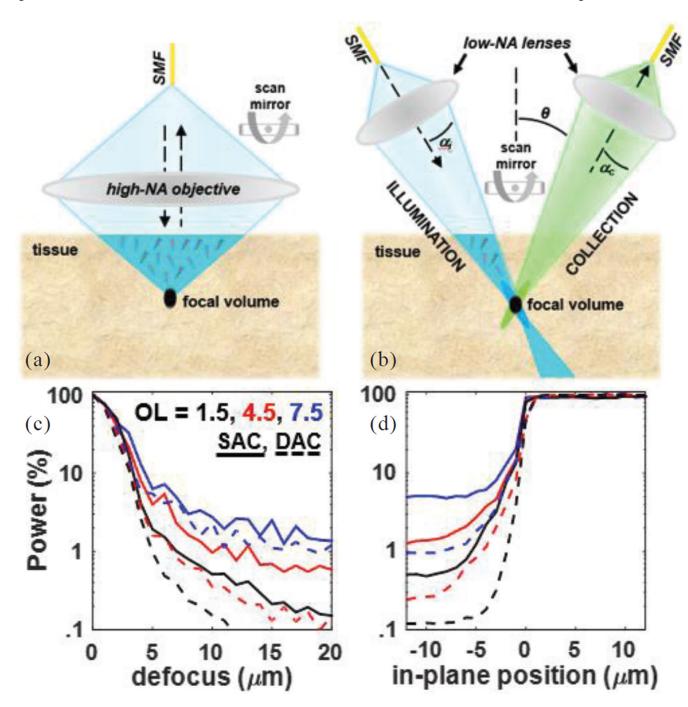


Fig. 1.

Confocal architectures and performance. (a) The single-axis confocal (SAC) architecture uses a high-NA objective to excite and collect fluorescence from tissue whereas (b) the dual-axis confocal (DAC) configuration uses spatially-separated low-NA lenses to excite fluorescence and collect light off-axis. Monte-Carlo simulations of DAC and SAC microscopes, with equivalent FWHM optical-sectioning thickness (axial resolution), show that the DAC architecture provides superior rejection of out-of-focus and multiply scattered light in turbid media both in the axial direction (c) and in-plane (d) over a range of optical

lengths (OL = $2 \mu_s d$ where μ_s is the scattering coefficient and d is the media's thickness). See text for additional details.

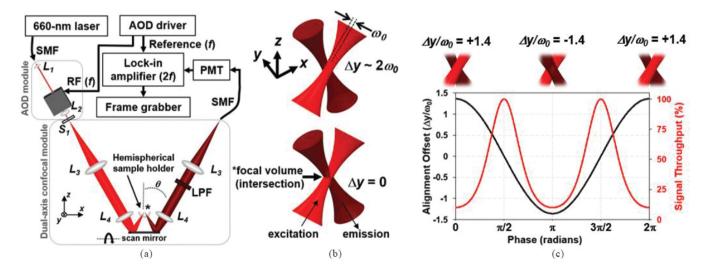
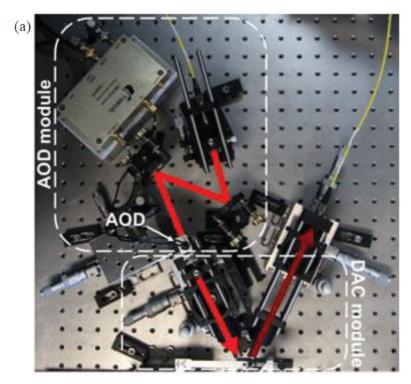


Fig. 2. (a) Electro-optical circuit of a MAD confocal microscope (* = focal volume). The AOD module collimates and scans a Gaussian laser beam over a small angular range on the order of one milliradian. The collimated light is focused to a point, which is relayed by the dual-axis confocal module to the imaging specimen to illuminate a volume of interest. A collection volume is confocally aligned to the illumination volume, which collects signal in a point-by-point manner as scanned by a galvonometric mirror. (b) Focal volume region where micron-scale alignment modulation generates strong amplitude modulation at the focus. (c) A simulation of the focal volume signal modulation for a modulation depth $y/\omega_0 = 1.4$.



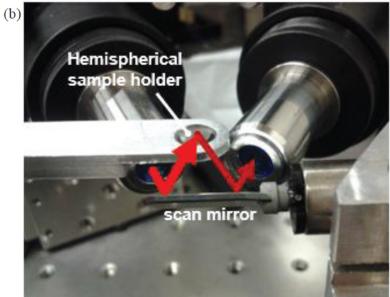


Fig. 3.Photograph of MAD confocal system. (a) Excitation and emission paths are indicated with light and dark red arrows, respectively. (b) The focal volume is formed by the intersection of the excitation and emission paths and is raster-scanned through the specimen, which rests on top of a hemispherical sample holder.

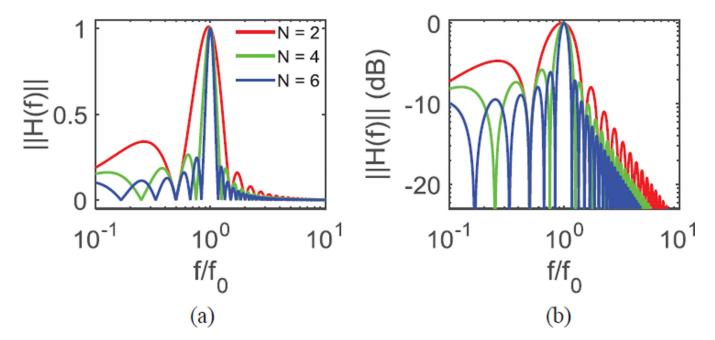


Fig. 4. Lock-in amplifier low-pass filter behavior as a function of number of integration cycles. (a) Linear and (b) log scales of the transfer function magnitude for a lock-in amplifier as a function of normalized frequency (f/f_0) and number of integration cycles (N).

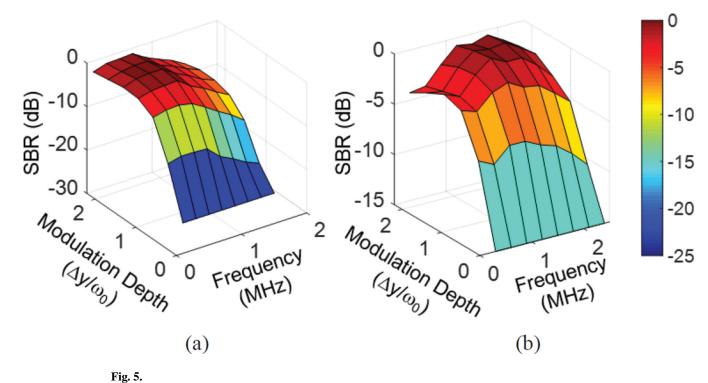


Fig. 5. Signal-to-background ratio (SBR) as a function of modulation depth and modulation frequency. The plots reveal the optimal modulation frequency obtained with a previous MAD system (a) and our newly optimized MAD system (b). With our previous system, a standard AOD was utilized with an input beam diameter of 0.99 mm ($1/e^2$), exhibiting an optimal modulation frequency of ~500 kHz at a modulation depth of ~1.8 (a). For the optimized MAD system, a beam-steered AOD was utilized (see text for details) with an input beam diameter of 0.32 mm ($1/e^2$), exhibiting an optimal modulation frequency of ~1.8 MHz at a modulation depth of ~1.8.

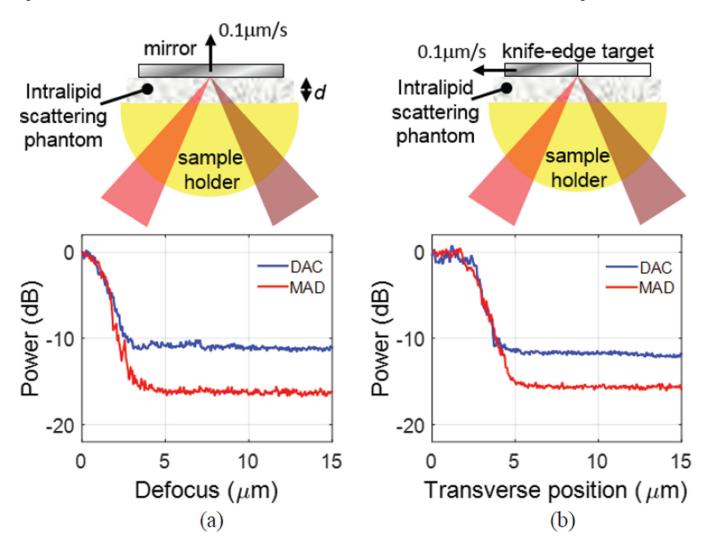


Fig. 6. Experimental results of optimized MAD confocal microscope performance in an Intralipid scattering phantom. (a) Axial resolution is preserved $(2.6-2.7 \, \mu m)$ while contrast is improved by $5-6 \, dB$. (b) In-plane resolution is maintained $(1.7-1.8 \, \mu m)$ while contrast improves by 4 dB.

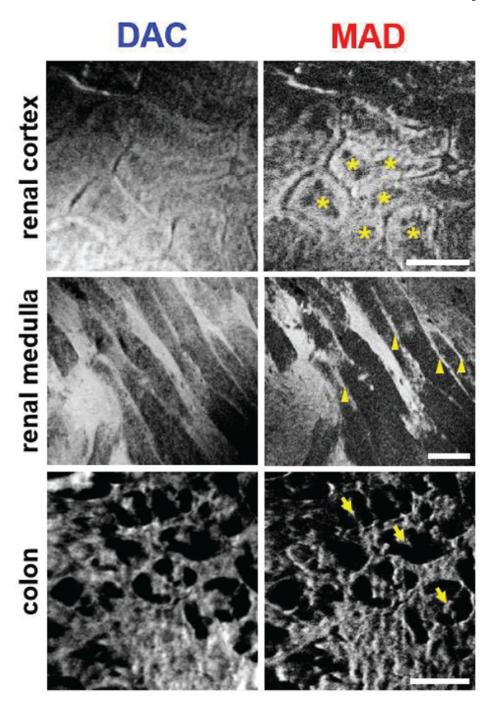


Fig. 7. DAC vs. MAD microscopy performance for fluorescently labeled mouse kidney tissues (fresh) imaged at 2 frames per sec (1.6-MHz pixel clock). DAC microscopy (left) exhibits limited contrast due to background signal contamination while MAD confocal microscopy extracts in-focus light more effectively and suppresses the background. Asterisks indicate proximal and distal convoluted tubules, triangles point to collecting ducts and loops of Henle and arrows indicate colonic crypts. Imaging depth $\sim 100~\mu m$. Scale bars = 75 μm .