Breaking Speed and Sensitivity Limits

Real-Time Diagnostics with Serial Time-Encoded Amplified Microscopy

High-throughput imaging is needed to identify rare diseased cells among a large population of healthy cells. When combined with microfluidics, this label-free cell identification method can revolutionize pathology, cancer screening, and potentially even stem cell therapy. Featuring optical image streaming and amplification, this new approach to imaging overcomes the technological and fundamental limits of the classic image sensor technology and may represent the solution.

Optical microscopy performed on stationary cells that are fixed on a glass slide, the so-called blood smear test, is the de-facto standard for diagnosis of disease in hematology and pathology. Here, microscopy performed on stained cells and tissue provides detailed images of their shape and internal structure that are used to identify diseased cells. While there has been breathtaking progress in microscopy in the past decade, emphasis has been overwhelmingly placed on improving spatial resolution, resulting in an impressive arsenal of nanoscopy tools that can break the diffraction limit of light [1, 2]. Mostly overlooked is the temporal resolution of imaging systems. High-throughput imagers are, however, needed to identify rare diseased cells among a large population of healthy cells during the early stage of disease progression. Such a technology will have other important uses; for example, it determines, in a minimally invasive way, whether a treatment is working in patients who are undergoing drug or radiation therapy.

An emerging and vital application is the detection of circulating tumor cells (CTCs) – the vanishingly rare tumor cells that circulate in a patient's blood and are forerunners of the metastatic stage responsible for 90% of cancer-related motalities [3]. In fact, a recent study has shown these rogue cells may be present even during the pre-metastasis stage [4]. But CTCs are exceedingly rare and hard to capture. To be sure, their

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population can number only a few parts per billion of normal blood cells. In fact, finding them is a problem that evokes the proverbial adage "finding a needle in the haystack." Without a doubt, detecting CTCs with good statistical accuracy requires a high-throughput technique that can sift through an enormous population of cells. A high-throughput microscopic blood imaging system can indeed provide a minimally invasive way to detect and monitor cancer and will usher in a new era in pathology. Indeed, we are increasingly observing the emergence of a growing population of either primary or metastatic cancer survivors further supporting the urgent need to establish real-time monitoring systems prior to the onset of clinical recurrence. We anticipate that cancer therapy will progress from destroying tumorigenic cells to ultimately seeking and destroying the cancer stem cell. Novel approaches, such as the STEAM technology, may enable this advance in 21st century medicine.

Flow cytometry

Such a high-throughput cell screening instrument would consist of a high-speed imaging system and flow cytometer. Flow cytometry is a powerful tool for analyzing millions of cells based on their shape, size, and biochemical properties. It is currently the workhorse of many applications such as cell counting in conventional hematology [5] and the study of cell diseases through immunophenotyping: the detection of proteins expressed by cells [6, 7]. In a flow cytometer, cells are funneled to the center of a flow stream that causes them to pass in a single column through a laser beam where they scatter light that is subsequently collected by photodetectors. By capturing the statistical distribution of the forward and side scattered light, the instrument classifies the cells based on their size and the granularity of their internal structure. The state-of-the-art flow cytometer can sift through a large population of cells at nearly 100,000 cells per second. Even higher throughput can be achieved by microfluidic channels with parallel cell flows [8]. Such high-throughput screening is highly valuable because every milliliter of human blood contains of nearly 5 billion cells (for reference, an average person's body has 5 liters of blood). Fortunately, most of these (at a ratio of approximately 1000:1) are red blood cells that are removed in typical blood test through a simple process called lysis . Therefore, the problem of identifying CTCs translates into finding diseased cells that appear as a few parts per 10^6–10^7 population of healthy cells.

Current flow cytometers perform cell counting and fluorescence spectroscopy [6, 7], but unfortunately they have no means to provide real-time microscopic images of cells because there is no imaging technology with a sufficient combination of speed and sensitivity. When the statistical distribution of scattered laser light suggests the presence of abnormalities in the cell population, a small sample of the blood is smeared onto a glass slide which is then manually inspected under a microscope. Unfortunately, this manual inspection is slow and at best can screen only a small

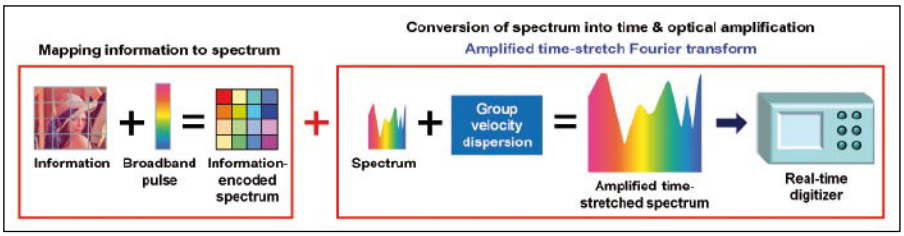

FIGURE 1: **Concepts of spectral encoding and amplified time-stretch Fourier transform.**

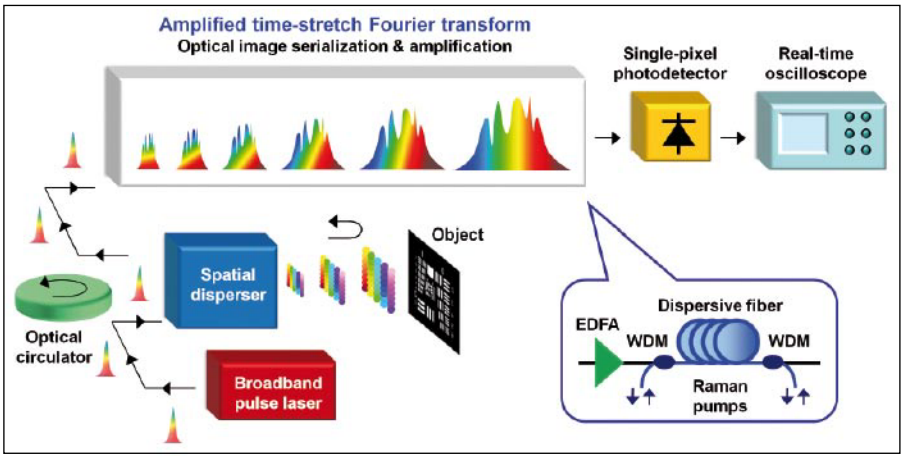

FIGURE 2: **Serial time-encoded amplified microscopy (STEAM).**

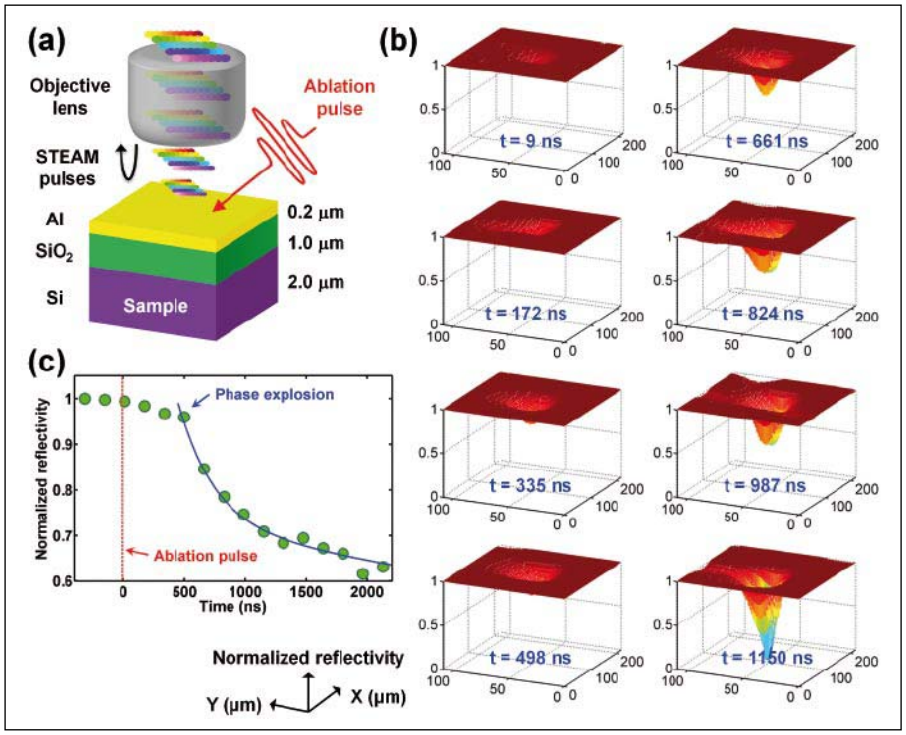

FIGURE 3: **Observation of laser ablation dynamics with STEAM. (a) Schematic of the experiment. (b) Real-time STEAM images of the laser ablation dynamics at a frame rate of 6.1 MHz corresponding to a frame repetition period of 163 ns with 440 ps shutter speed. (c) Evolution of the normalized reflectivity map of the sample that indicates the phase explosion effect.**

fraction of the cell population. Therefore, it is highly prone to statistical error. In other words, rare cancer cells cannot be detected this way. A negative test result is by no means an indication of the absence of diseased cells in a patient's blood. A high-throughput imaging flow cytometer will offer far higher statistical accuracy by screening through a much larger volume of blood than in today's blood smear test.

Limitations of CMOS and CCD cameras

While high-end image intensified CMOS cameras are now able to perform imaging at a speed approaching one million frames per second, they require high-intensity illumination to overcome the loss of sensitivity at high speed. After all, during short frame intervals, fewer photons are collected, leading to a reduction in sensitivity. CMOS and CCD cameras are not well suited for high-speed microscopy because when focused onto a micrometer size focal spot, the high-intensity light can alter or damage the cell. A critical limitation that makes them unsuitable for imaging cells in flow is the relatively long shutter speed (hundreds of nanosecond) that results in blurring and loss of resolution during the high-speed flow. Another limitation on frame rate is the time needed to download the image from the array of pixels (readout time). To achieve high frame rates, the number of pixels that are employed must be reduced in a process known as the partial readout [9]. The penalty is that image resolution is lost at high frame rates.

If realized, an imaging flow cytometer can transform hematology as it will eliminate the need for manual inspection of blood samples under a microscope. Also, it will make possible detection of rogue cells such as CTCs. Indeed, its potential impact is great and is being recognized as evident by the recently introduced imaging flow cytometer by Amnis Corp. [10] a system that provides real-time fluorescence images of cells, but unfortunately, at 100–1,000 times slower flow rate than in a conventional flow cytometer, which has high flow rate, but no imaging capability. Nevertheless, because of the immense potential of imaging flow cytometry, it has been adopted by the medical research community.

High-throughput Real-time Diagnostics

We have recently demonstrated an entirely new approach to imaging that has high throughput and is promising for biological and industrial applications [11]. It belongs to a class of fast real-time instruments that perform a two-step process (Figure 1). In the first step, the information to be captured at a high frame rate is stamped onto the spectrum of a broadband optical pulse. This information can be a fast (wideband) electrical waveform such as that in a communication network, the spectroscopic signature of a material or process, or the image of an object. In the second step, the spectrum, and hence the information, is mapped into a time-domain serial stream whose amplitude modulation is the information of interest. At the same time, the stream is optically slowed down by a sufficient amount so that it can be captured with an electronic digitizer such as a digital oscilloscope.

This class of instruments can capture fast dynamic information, such as images, in real time because the timescale of the information is optically slowed down before it encounters the photodetector and electronic digitizer. It creates a wide range of real-time instruments that operate at speeds that are beyond the reach of their conventional electronic-based counterparts. They include analog-to-digital converters, spectrometers, and imaging systems. The work in creating such instruments began in the 1990's with analog-to-digital converters that achieve large real-time bandwidth by slowing down fast electronic waveforms using a dispersive fiber optic link [12, 13] and evolved into spectroscopy [14, 15] and imaging [11, 16, 17].

Serial Time-Encoded Amplified Microscopy (STEAM)

The principle of STEAM is the mapping of a multi-dimensional image into a 1D serial time-domain optical waveform that is optically amplified and then converted to an electronic waveform by a photodiode and

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finally digitized (Figure 2). This is done by first encoding the spatial coordinates of the object onto the spectrum of a broadband pulse with a spatial disperser. The optical source is a mode-locked femtosecond fiber laser with the centre wavelength 1,590 nm, bandwidth 15 nm, pulse energy 82 pJ, and pulse-repetition rate that could be adjusted from 6 MHz to 37 MHz by pulse picking. For 1D imaging, a diffraction grating or prism is used as a spatial disperser while for 2D imaging, a pair consisting of a diffraction grating and an orthogonally oriented virtually-imaged phased array is employed. The 2D disperser possesses a groove density of 1,200 lines per millimetre and a virtually imaged phased array with a free spectral range of 67 GHz and a linewidth of 550 MHz. These specifications may vary as they depend on the available optical bandwidth and number of pixels that is desired. The image encoding occurs when the spatially-dispersed pulse or rainbow is reflected off the object, after which it returns to the spatial disperser where the different frequency components of the pulse are recombined. An optical circulator directs the pulse into a novel optically amplified time-stretch Fourier transformer realized using an optical fiber with high temporal (group-velocity) dispersion and low loss [18]. While propagating in the fiber, each frequency component of the light (hence, each image pixel) experiences different time delays, causing pixels to walk away from each other and spread into a 1D data stream. In addition to transforming the optical spectrum into time, the dispersive fiber simultaneously amplifies the image because it is pumped to induce stimulated Raman amplification. The spatial image stamped onto the spectrum now appears as a serial sequence in time and is captured with an analog-to-digital converter such as a digital oscilloscope.

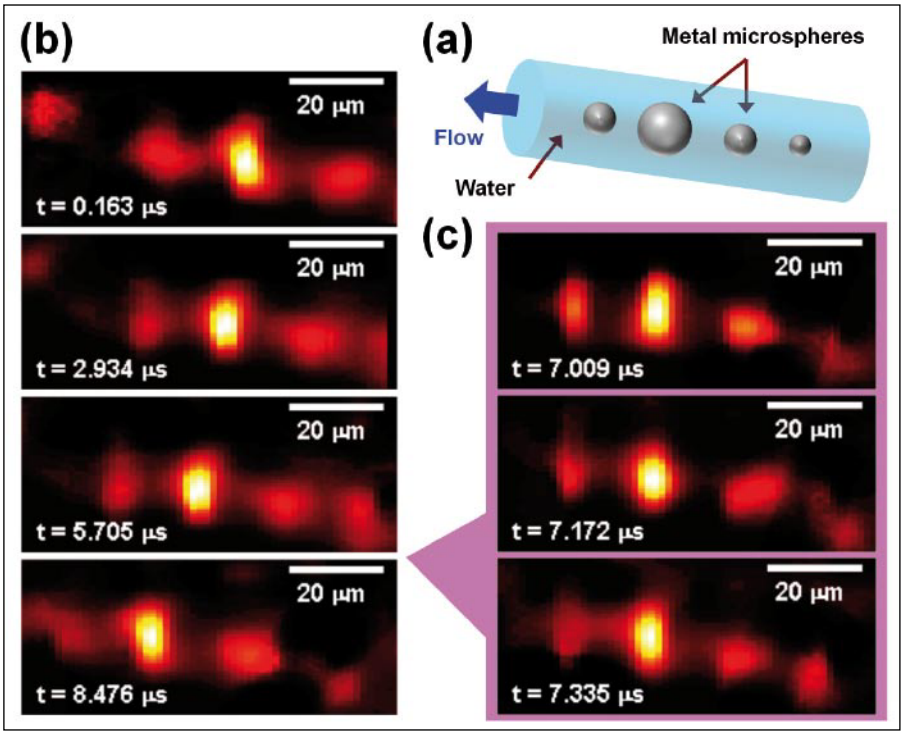

FIGURE 4: **Observation of ultrafast microfluidic flow with STEAM. (a) Laminar flow of water-suspended metal microspheres with various diameters in a hollow fiber. (b) STEAM images of the flow showing one out of every seventeen snapshots for clarity. (c) Consecutive STEAM images of the flow with a frame resolution of 163 ns with 440 ps shutter speed.**

The key technology limitation in any high-speed imaging or sensing system is whether the digitizer has sufficient bandwidth, or equivalently, whether its sampling grate – which has to be at least twice the bandwidth – is large enough (a requirement called the Nyquist sampling criterion). Because a higher stretch factor requires larger group-velocity dispersion that is accompanied by higher loss, the ability to stretch the pixel stream in time is critical as it overcomes the bandwidth limitation of the digitizer by slowing the image without loss. A more fundamental limitation in high-speed imaging and sensing is the loss of sensitivity as mentioned above. At high frame rates, fewer photons are collected within the short integration time, leading to the loss of signal. Optical image amplification is what allows STEAM to operate at millions of frames per second at shutter speeds of 100's of picoseconds, and with very low power (only a few mW) of optical illumination. In fact, in experiments described below, a net optical image gain of 25 dB – more than 300 times – was achieved. Without the amplification of the image in the optical domain, the loss of signal would have to be compensated with high-intensity illumination. For biological applications where the illumination is focused onto a microscopic field of view, this can lead to damage or destruction of the sample being studied. With STEAM, it is possible to obtain images at 10's of MHz frame rates while minimizing damage to the biological sample. Without its image amplification, the image would not be visible at such high frame rates because the signal would lie below the thermal noise of the fast photodetector. Animated movies that illustrate the functionality of STEAM for 1D and 2D imaging can be viewed on the internet at sites [19] and [20], respectively. The performance of STEAM as it relates to its optical resolution and how it depends on the specifications of the optical and electronic components is described in Ref. [21]. Because cells are mostly transparent, a phase contrast mode of STEAM is preferred to avoid the use of contrast reagents. Such a system has already been demonstrated at UCLA [22].

To demonstrate the ultrafast real-time imaging capability of STEAM, we successfully observed the dynamics of laser ablation with the imager. Laser ablation is a ubiquitous technology that is used in laser surgery, laser cutting and micromachining, and laser-induced breakdown spectroscopy. As shown in Figure 3a, a powerful mid-infrared pulse laser was focused at an angle onto a test sample while the imaging pulse train of STEAM was incident onto the surface of the sample at a normal angle. Figure 3b shows the images of the phenomenon obtained by STEAM at a frame rate of 6.1 MHz which corresponds to a frame repeti-

tion period of 163 ns. STEAM captured, in real time, the entire frame sequence corresponding to the dynamics (laser-induced mass ejection) caused by the single ablation pulse. Further analysis of the surface reflectivity change shows a finite time-delay between the pulse excitation and the sudden decrease in surface reflectivity caused by the ejection of material from the sample (Figure 3c). It is a signature of the phase-explosion effect that is the hallmark of laser ablation and firmly establishes the ability of STEAM to monitor fast dynamical processes in real time. To be sure, such high-speed monitoring of laser ablation dynamics in real time is extremely valuable because when combined with a feedback loop, laser power can now be adjusted in real time in such a way to dynamically adjust the power and exposure time in order to optimize the results of laser cutting and micromachining while minimizing collateral damage.

As a further proof of STEAM's capability, we used it to monitor ultrafast microfluidic flow. As shown in Figure 4a, we prepared laminar flow of water-suspended metal microspheres with various diameters ranging from 10 – 30 µm in a hollow fiber with a diameter of 50 µm. STEAM captured a total of 3,000 frames in real time (limited only by the memory of the real-time oscilloscope). Figure 4b shows one out of every seventeen snapshots for clarity, whereas Figure 4c shows consecutive frames with the frame resolution of 163 ns. The flow of metal microspheres from right to left is clearly observed and shows a velocity of 2.4 m/s. As a benchmark, the state-of-the-art flow cytometer operates at 1–5 m/s. This is the first time that such ultrafast microfluidic flow has been observed in real time with such a fine temporal resolution. STEAM is expected to be a valuable tool for imaging flow cytometry.

Optical coherence tomography (OCT) [23, 24, 25] is an optical reflectometry method that provides images of near-surface internal structure of semitransparent material such as biological tissue. In OCT, light reflections from discontinuities in the refractive index along the depth (axial) dimension are converted to intensity changes using an interferometer. For each wavelength, constructive interference leading to maximum intensity occurs for different depths. This process encodes the sample's depth profile onto the optical spectrum. In the OCT version of the STEAM technology, this profile is first mapped onto the spectrum of a femtosecond optical pulse. Then using the amplified time-stretch Fourier transform, the spectrum is simultaneously amplified and converted into a time-domain serial stream whose amplitude modulation is the depth profile of the sample [17]. As with STEAM, the stream is slowed down such that it can be captured with a real-time digitizer such as a digital oscilloscope. The OCT-STEAM has achieved a record scan rate of 37 MHz [17]; 100 times faster than conventional high speed OCTs. One of the areas in which high-speed OCT will be needed is volumetric imaging where numerous axial scans must be performed at many points on the surface to capture the 3D image. This technique is expected to be useful for industrial applications where high-speed imaging capability is required to monitor a large number of parts.

Conclusion

In summary, we have described a new method of imaging that performs fast microscopy in realtime and with very low illumination intensity. It has the potential to find rare cancer cells among a large population of healthy cells for early stage detection of cancer prior to the onset of clinical disease. In addition, it may find industrial applications where real-time monitoring and dynamic control of manufacturing processes is needed to optimize them. Finally, a near term application can be an ultrafast barcode reader in pharmaceuticals, packaging, and distribution industries.

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