LA-UR- 99-954

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Title: VIRTUAL PINHOLE CONFOCAL MICROSCOPE

Author(s): John S. Geroge, David M. Rector, Douglas M. Ranken, P-21

Bret Peterson, SciLearn Inc.

John Kesterson, VayTech Inc.

Submitted to: TECHNICAL REPORT

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Los Alamos National Laboratory Technical Report
LAUR:

Virtual Pinhole Confocal Microscope

John S. George, David M. Rector, Douglas M. Ranken,
Bret Peterson[1] and John Kesterson[2]

Biophysics Group, Los Alamos National Laboratory; [1] SciLearn Inc.; [2] VayTech Inc.

Abstract: Scanned confocal microscopes enhance imaging capabilities, providing improved contrast and image resolution in 3-D, but existing systems have significant technical shortcomings and are expensive. *Researchers at Los Alamos National Laboratory have developed a novel approach—virtual pinhole confocal microscopy—that uses state of the art illumination, detection, and data processing technologies to produce an imager with a number of advantages:* reduced cost, faster imaging, improved efficiency and sensitivity, improved reliability and much greater flexibility. Work at Los Alamos demonstrated proof of principle; prototype hardware and software have been used to demonstrate technical feasibility of several implementation strategies. The system uses *high performance illumination*, patterned in time and space. We have built functional confocal imagers using video display technologies (LCD or DLP) and novel scanner based on a micro-lens array. We have developed a prototype system for *high performance data acquisition and processing*, designed to support realtime confocal imaging. We have developed algorithms to *reconstruct confocal images* from a time series of spatially sub-sampled images; software development remains an area of active development. These advances allow the collection of high quality confocal images (in fluorescence, reflectance and transmission modes) with equipment that can inexpensively retrofit to existing microscopes. Planned future extensions to these technologies will significantly enhance capabilities for microscopic imaging in a variety of applications, including confocal endoscopy, and confocal spectral imaging.

Introduction

For almost 400 years, optical microscopy has been the principal method for the study of biological structure at the cellular level. Over the past century the application of photography has allowed the capture and distribution of microscopic images to a wide audience. The advent of electronic imaging is producing another revolution in scientific microscopy, that is redefining the frontiers of speed, temporal and spatial resolution, sensitivity, and perhaps most importantly, the capacity for quantitative photometric, spectral, and geometric measurements.

Confocal microscopy systems offer a number of advantages for quantitative imaging including improved image contrast, resolution, and limited depth of field. The limited depth of field of scanned confocal microscopes has supported applications not previously feasible, such as the optical sectioning and volumetric reconstruction of complex subcellular structures, or mapping the spatial and temporal distributions of intracellular ions. Conventional confocal systems incorporate several key functional subsystems: a system for scanned illumination; a spatial filter or aperture in the focal plane of the detector to reject out of focus light, and a single channel or imaging detector. Imaging optics bring the illumination and imaging paths into focus at the same location within a sample—hence the term "confocal". Most existing commercial systems are designed for epifluorescence imaging, allowing scanning and aperture functions for illumination and imaging to be performed by a single piece of hardware. Initial commercial confocal microscopes appearing in the mid-1980s utilized laser illumination and single channel photomultiplier detectors. Although a number of important technical advances have emerged over the intervening years, most confocal systems remain complex, inflexible, slow and expensive ($50-250k).

Researchers at Los Alamos National Laboratory have developed a novel approach for confocal microscopy that exploits available illumination, detection, and data processing technologies to produce an imager with a number of advantages: reduced cost, faster imaging, improved efficiency and sensitivity, improved reliability, and much greater flexibility. This system has three key components: 1) an electronically or electro-mechanically scanned

illumination subsystem, 2) an area sensor such as a CCD imager, and 3) a "virtual pinhole"—synthetic aperture—constituted during image processing after sensor readout. Our approach provides the core technologies for a family of microscopic imaging methods that will significantly advance the utility of optical microscopy for clinical applications and basic research. The initial focus of our work has been the development of a low cost system for confocal imaging suitable for retrofit integration with conventional research microscopes. However, our approach also offers simple and effective solutions for transmission confocal imaging, a practical approach for confocal imaging through an endoscope and for high performance confocal spectral imaging.

Confocal Principle and Practical Instruments

A conceptually simple embodiment of a confocal microscope is illustrated schematically in figure 1. This system is configured for transmission imaging. Light from a source is transmitted through a pinhole. Light from the pinhole is brought to a point focus within the sample volume by imaging optics. Light emerging from the sample is brought to focus on a pinhole aperture by a second set of imaging optics. This aperture acts as a spatial filter for imaging, rejecting most of the light that did not pass through the confocal point in the sample volume, thereby reducing image contributions from light scattered or refracted from other locations within the medium. The detector for such a system typically consists of a single channel device such as a photodiode or photomultiplier. In order to collect an image, it is necessary to scan the illumination and detection subsystems. However, the technical requirements for coupled electromechanical scanning to preserve the required optical alignment between illumination and imaging pathways are formidable. Most practical embodiments of a transmission confocal system utilize mechanical scanning of the sample so that the imaging elements do not move, however scanning in such systems is very slow.

The conventional configuration for confocal imaging in reflected light or fluorescence modes is illustrated in figure 2. This configuration employs the same imaging optics and often the same pinhole aperture for both the illumination and imaging paths. This arrangement simplifies optical alignment and facilitates optical scanning, by mechanical movement of the aperture, or by shifting the image of the aperture by mirrors in the optical path. In many designs, the scanning of the illumination path and the "descanning" of the imaging path are accomplished with the same set of mirrors. Image acquisition in most existing systems is relatively slow, which complicates preliminary inspection of a sample and focusing.

Spinning disk confocal systems are a useful implementation of the principles illustrated in figure 2. In such systems the scanning and aperture functions are embodied in a opaque disk containing a pattern of optically transmissive pinholes. The disk is rotated at high speed, and the pinholes are arrayed so that during a revolution of the disk, the entire sample image plane is illuminated. Each pinhole serves as the aperture for both illumination and imaging paths. Because a number of locations within the sample are scanned in parallel, the detector typically consists of a spatially resolved area detector such as a video camera, although direct viewing by eye is also possible. These systems can significantly enhance the speed, flexibility and ease of use of confocal microscopy. However, they typically suffer from low sensitivity (illumination is inefficient since most light is absorbed by the opaque regions of the disk) and from reflection from the disk surface. The systems are relatively bulky, and the gyroscopic nature of the spinning disk inhibits its application for compact portable imaging systems such as endoscopes.

Another strategy for high performance imaging is to use an illumination source configured as a line instead of as a point, with a slit aperture instead of a pinhole. Such scanning systems are considerably simpler and faster than mirror systems used for point scanned illumination, since the pattern of illumination is scanned in only one dimension. This method employs parallel detection using a specialized detector such as a linear CCD, which can considerably reduce imaging time. This enhancement of performance comes at some compromise of imaging quality. In a point scanned imager, the pinhole aperture rejects light from any apparent sample location displaced from the confocal point. In a line scan configuration, out-of-focus light that happens to fall onto the slit aperture contributes to the image, however in practice the degradation of confocal image quality is not unacceptably severe. In a spinning disk configuration a line-scanner produces a brighter image, though additional light is presumably non-confocal.

An alternative approach developed by Greenberg and colleagues at NIH, in principle offers higher performance, since the need for mechanical scanning is eliminated. The system scans a laser in two dimensions, using a pair of *opto-acoustic beam steering* devices. The detector is based on an *image dissector videcon-* a television detector tube that allows random access readout of the tube photocathode at user defined coordinates (defined by a pair of voltages). However, like most other tube-based video detectors, the image dissector has been rendered obsolete by the advent of solid state video devices, and a commercial version of this confocal instrument has not been produced.

Several other developments are important to define the contemporary technical context of confocal imaging. Technical improvements in available *scanning mirrors* (in particular the use of resonant galvonometers) have allowed faster imaging using conventional confocal optical configurations. Other designs employ *optoacoustic beam steering* in addition to a mirror scanner to achieve very fast scan rates, but existing designs sacrifice image quality. Optoacoustic beam steering devices do not properly descan the returning light in fluorescence applications, so that the optical properties of the system are similar to a linescan confocal device. In another patented design, an *optical fiber pinhole aperture* is used for illumination and detection. This approach greatly simplifies the linkage between a conventional microscope and confocal illumination and detection subsystems, and has been used to build a prototype confocal endoscope.

Our approach to confocal imaging *operates on the same principles as existing confocal imagers, but realizes the necessary functional subsystems in a novel way.* This technical approach is the basis of a Los Alamos patent application currently being considered by the US patent office. Our approach enables a number of possible applications and generates a number of advantages.

The first key subsystem is a system for *electro-optically scanned illumination.* Several classes satisfy our basic requirements for speed and precision. Scanned illumination is achieved by the use of spatial light modulators *(SLMs)—de*vices that can be electronically driven to produce time-varying spatial patterns of illumination. We have explored the use of display devices used in video projectors. Most computer compatible video projectors now employ Liquid Crystal Devices (LCDs). Texas Instruments recently introduced Digital Light Processor (DLP) technology, based on an electronically addressable micro-mirror array. Our work has shown that confocal imaging with these technologies is feasible. The technologies are relatively mature, flexible and cost-effective, and the design of these devices allows construction of simple, compact illuminators. Contrast and pixel image quality were disappointing in a miniature silicon LCD we evaluated in early work. However, significant improvements in video projector performance over the past two years have produced LCD technology viable for this application. Although LCD and DLP technologies are extremely flexible, they waste light. We have shown that microlens arrays can be used to produce a grid of illumination points, with high efficiency and good optical characteristics. We have built a prototype scanner based on this technology using a precise stepping motor, however piezoceramic actuators, and galvanometer systems may have advantages for this application.

The second key technical strategy is the use of a *"virtual pinhole"—a synthetic aperture* constituted after readout of a solid state video imaging array-- typically a charge-coupled device (CCD), charge injection device (CID), photodiode array camera or similar device. In general the synthetic aperture is implemented in software on the host computer or in a specialized digital signal processing engine after digital image acquisition. These computations predict how much light would have passed through a physical aperture located in the focal plane of the microscope. We have demonstrated several workable algorithms, including some that are very efficient to compute. Because functional parameters are implemented in software, it is possible to adjust key parameters (such as effective aperture size) to optimize image tradeoffs, even after the basic data are acquired.

In order to increase the performance and efficiency of the system it may be useful to employ more sophisticated procedures to read out the sensor array. Most solid state cameras are able to skip (dump) entire video lines or to bin pixels within video lines at much higher rates than are required for standard readout. This approach allows mush faster image acquisition and more efficient use of image memory on the acquisition board. However, even without such methods, we believe that existing CCD, data acquisition and signal processing technologies will allow the acquisition and reconstruction of confocal images at rates suitable for continuous video display

Thus, the complete system consists of imaging optics, typically supplied by a standard research microscope, a system for scanned illumination, a standard or high performance solid state video camera, and a computer system for image acquisition, scan control, and image reconstruction.

The system has a great deal of flexibility, not available in other confocal systems. The detector can be operated as a conventional video camera, allowing easy focusing and survey of the microscope image field. The imager can be readily configured to operate in transmitted light modes as well as the reflected light or epifluorescence modes offered by most confocal microscopes. A number of image contrast mechanisms can be digitally synthesized: e.g. transmitted light darkfield (for scattered light) can be produced by changing the convolution kernel used to create the synthetic aperture. Multiple images can be computed from a single data set to optimize image data for a given application.

Our system provides confocal imaging capabilities to existing research microscopes, for little more than the cost of a conventional microscope with digital video capability. The system will be developed for confocal transmission imaging, since this is an under-served application. Component systems will be designed for flexibility; reflected light and fluorescence imaging capabilities use the same components. The imaging system provides a family of instruments, optimized for different applications: a spectrally-resolved microscopic imaging system, that produces a complete spectrum for each image pixel; a high performance system with extended sensitivity, speed, and dynamic range; a confocal and spectroscopic endoscope system—rugged and light weight because no moving parts are required. We anticipate that advanced systems based on these ideas will be developed in future work.

Technical development

We have produced engineering prototypes of each of the key systems required for the virtual pinhole confocal microscope. Using off-the shelf components we have been able to acquire and reconstruct confocal images in less than two seconds, which is comparable to the acquisition time in many existing confocal microscopes. Both on technical grounds and based on market considerations, we plan to achieve confocal imaging at continuous video rates (30 frames per second). In order to achieve this goal, we will need to illuminate, acquire and process raw frames at 480 frames per second from a 256^2 imager. There are no fundamental obstacles to achieving the enhanced performance eventually desired.

Illumination:

Confocal microscopy depends on scanned illumination. In conventional confocal imaging this is most often accomplished by electromechanical scanning of a laser beam and image light is detected with a single channel device such as a photomultiplier. Like the spinning disk confocal systems, our approach employs a 2D detector and illuminates the image field with a number of spots in parallel, separated sufficiently to minimize crosstalk. We have developed several approaches for high performance illumination, patterned in time and space. Our general strategy is to use a Spatial Light Modulator (SLM) under computer control. We are focused on three technologies which can achieve the required performance: LCDs, DLPs, and scanned microlens arrays (MLAs).

The illumination subsystem will be housed in a compact box that sits next to the microscope (for transmission imaging applications) or can replace the lamphouse typically employed for epifluorescence microscopy. Using transmissive scanning devices (LCDs and MLAs) a much smaller unit has been developed that is inserted into the microscope optical path like a filter. The prototype system was designed to allow the use of an internal light source (such as a projector lamp or microscope illuminator), but will also allow the use of external sources, with light introduced through fiber optic light guides. This subsystem incorporates relay optics to deliver the illumination image to a conjugate image plane of the microscope and to allow adjustment of magnification. In the configuration for transmission imaging, the light path from SLM to objective will be enclosed.

Liquid Crystal Displays (LCDs) In earlier trials, LCD pixel quality and contrast were also disappointing, however recent improvements in the performance of video projectors suggest that the technolgy of miniature silicon based LCDs has matured considerably. Given the potential advantages of a programmable transmissive illuminator, we plan to reevaluate LCD technology.

Digital Light Processor (DLP) Preliminary work with DLP devices has identified limitations with the speed and flexibility of the drive/control system used with the engineering evaluation device. Therefore, during Phase I work we will evaluate the feasibility of direct drive of the SRAM framebuffer used to control the micromirror array. This will begin with consultation with TI to develop a better understanding of the limits of the device and the requirements to control it. Based on this exercise we expect to either modify the evaluation board, or design and fabricate a new drive circuit to allow the DLP to be driven by the data acquisition subsystem.

Microlens Arrays (MLAs): Our previous studies have shown that MLAs can be used to produce a grid of illumination points, with high efficiency and good optical characteristics. We have built a workable scanner based on this technology. We will continue to evaluate several scanning systems, including stepping motors, piezoceramic actuators, and conventional galvanometers. Primary challenges are to achieve adequate speed and precise and reproducible positioning.

In order to demonstrate the proof of principle, Los Alamos researchers have built a series of research prototype instruments based on the approach outlined above.

To begin testing the use of solid state area detectors for confocal microscopy, we implemented an initial prototype microscope using a consumer-grade CCD camera as the detector. For the preliminary tests, the beam was scanned mechanically by moving a lens through a collimated beam. The lens focused the light to a point at a plane corresponding to the focal plane of the CCD imager. A diagram of the system is shown in figure 5A. This set up allowed the properties of the illumination spot relative to the CCD to be adjusted independent of the microscope objective and thus independent of the magnification. A microcomputer was used to control the scanning of the illumination spot and to collect the image from the CCD. In order to test the *depth of field*, one-dimensional scans were made across a high-contrast target (a line on a microscope stage micrometer) at different depths. Equivalent cross sections were measured from conventional bright field microscopic images collected with the same imaging configuration and components. In these reflected light images, light was brought through the microscope objective via a beamsplitter. Figure 5B shows cross sections through depth of the signal-producing feature with the images. These cross sections give a clear demonstration of the narrow depth of field and the high contrast produced by the prototype confocal imager.

Confocal Image Collection: Although the experiment described above established that the virtual aperture system performed optically as a confocal imager, clearly this system would not serve as a practical microscope. Scanning was slow and relatively imprecise, providing none of the performance advantages of parallel illumination and readout that are among the principal motivations of the proposed method. In order to explore possible technical strategies, we have set up a series of research prototype instruments. Each has been constructed on an optical bread board employing commercially available subsystems.

The second prototype was based on an *LCD based video projector* driven with a standard NTSC video signal. A portion of the light emerging from the projection lens was collected using a large aperture photographic lens, and was brought to a focus at a conjugate image plane of the microscope. The imager was a miniature CCD camera and 8-bit digital images were acquired using a microcomputer resident framegrabber. Although it was possible to collect images with this system, a number of problems were identified. Resolution of the video projector (which ultimately limits resolution of the imager) was poor; optical coupling was sub-optimal with poor efficiency, significant internal reflections; and low contrast; and the system was slow and suffered from the interleaved video field format of the NTSC projector. The video projector itself was comparable (or larger) in bulk than many existing confocal microscopes.

We explored the use of raw *LCD devices* in an effort to address a number of the perceived problems of the projector based system. The comparatively large format of most existing LCDs at the time (>3 inches diagonal) posed significant problems for uniform illumination and efficient optical coupling, given the requirement to form an image of the array comparable to the size of the CCD. However, a miniature silicon-based LCD produced by Kopin, allowed us to build a working transmission imager; an image is illustrated in figure 6A. The simplicity of the design was appealing; we were able to use the microscope's built-in light source and optics for brightfield imaging. However, the contrast of the imager and the optical quality of the projected pixels was disappointing.

Also, the LCD system was inadequate for fluorescence imaging, and was suitable for reflected light imaging only with highly reflective samples such as integrated circuits. These problems lead us to investigate other strategies for scanned illumination. However, over the last 1-2 years, a number of high resolution LCD based projectors incorporating miniature silicon based LCDs have been introduced for computer generated presentations. These address many of the problems with earlier devices.

Over a similar time-frame, Texas Instruments has introduced *Digital Light Processor (DLP) technology*, and initial video projectors incorporating this technology have appeared commercially. After executing a non-disclosure agreement with TI (a process that required considerable time and persistence), Los Alamos researchers have acquired two engineering evaluation units employing the micromirror devices. One is the compact core of a video projector unit, incorporating the light source and all necessary optics and electronics. This unit is less than a quarter of the size of the 1991 vintage LCD projector used in previous work, and employs a much smaller projection lens, simplifying optical coupling. However, the device as provided requires a special VGA card that produces a digital video signal. The projector uses a spinning filter wheel to provide color projection capability, but this system interacts with the video acquisition to produce a temporal interference pattern. Finally, the vibration set up by the cooling fan created serious problems for our prototype microscope.

The contrast and pixel quality of the DLP system were superior to the Kopin LCD. The device also avoids the inefficiency associated with the polarizers required for LCD projection. In an effort to avoid the problems observed with the DLP projector (noted above), we obtained an evaluation board consisting of the DLP device and driver electronics without the optics or light source. This system is used in an existing prototype; an image acquired with this system is illustrated in figure 6B. Although we had initial problems with poor contrast, improved collimation of the illumination light largely addressed this concern. The spatial uniformity of illumination and the quality and flexibility of optical coupling within the illumination path were improved with relatively straightforward optical design changes. In one prototype we employed a coherent fiber optic image guide to couple the image from the DLP to the illumination objective. This as done for convenience, to help isolate vibrations, and to test the feasibility of this approach for scanned illumination in a confocal endoscope. Although the approach is workable, it does introduce some spatial degradation of the illumination pattern that is unnecessary in a microscope configuration. We also have encountered problems with environmental vibrations, but improved mechanical design largely addressed this problem.

The most significant limitations of this system arise in two areas. First, the existing electronic drivers are geared toward gray-scale images presented at computer video rates (i.e. 60-70 Hz). The application specifications for our targeted hardware configuration requires binary (B&W) illumination and much higher frame rates. Our preliminary studies of the DLP suggest that it should be possible to drive the unit directly by writing digital data to the SRAM memory controlling the state of the micromirror array—bypassing the video encoding circuitry to achieve the desired performance. A second problem is more integral to our application of the DLP or LCD devices. In our usual scanning method, only a small fraction of the total number of pixels is illuminated at any given time, typically on the order of 1 in 25. Separation between illuminated spots is maintained to avoid crosstalk between scanned points. However, this means that 96% of the available light is wasted. For some applications this is of little concern and the flexibility of programmable illumination and solid state devices justifies the tradeoff. However, for light-limited applications (such as typical fluorescence microscopy), this is a significant problem.

In an attempt to address this problem, we have build a scanning system based on a *microlens array*. This is a small replicated optical element consisting of an array of microscopic lenslets, typically formed in a regular (square or hexagonal) grid. Lenslet diameters (and thus the pitch between elements) is on the order of ten to hundreds of microns. As in macroscopic convex lenses, if collimated light is passed through the lenslet, the light will be focused to a point at the focal length of the lens. With an array, virtually all of the light is focused in a grid of illuminated points. Each of these points is a much better approximation to a Gaussian profile than is achieved with the LCD or DLP projection arrays. The illumination pattern can be scanned by mechanical translation of the microlens array, over relatively short distances (comparable to the pitch of the lenslet array).

Figure 7 illustrates a novel method for scanned illumination using such a lens array. The lenslets are illustrated by the larger circles in figure 7; focused points of illumination are illustrated by the smaller circles. Figure 7A illustrates partial sampling of the image plane produced by translation of the microlens array in two dimensions. The illustrated grid is produced by three positions of the microlens array. By additional translations in two dimensions, the entire image plan can be sampled. Figure 7B illustrates an alternative scanning geometry achieved by tilting the microlens array. This configuration achieves high density sampling by translation along a single dimension. The most obvious strategies for scanning the array include rotary or linear stepping motor drives (with performance comparable to those used in hard-disk drives), galvanometer drives; or piezoelectric or ceramic devices (which may be suitable since the load is low and limited translation is required). Although in principle, a spinning lens array could be used for scanning, this introduces a number of technical challenges that we wish to avoid at this stage of the development effort. The plan to employ mechanical scanning reintroduces concerns about system reliability, vibration, and the resolution and reproducibility of positioning. However, the performance requirements are comparable to modern mass storage devices and we can use well developed technical solutions.

Data Acquisition and Processing:

Our approach can achieve high quality and high performance imaging to compete with the best of conventional confocal microscopes. Although our method can be implemented with commercially available video acquisition hardware and general purpose computers, there are a number of technical and commercial advantages to developing a specialized data acquisition and processing unit. We will continue to implement and evaluate advanced algorithms for image reconstruction. We anticipate that different algorithms will be optimal for different requirements; an algorithm for realtime reconstruction and display works differently from an algorithm designed for optimal image quality or depth resolution

We are building a redesigned, high performance data acquisition and processing system, designed to support near-realtime confocal imaging with the virtual pinhole microscope. The system will consist of a dedicated Intel/PCI motherboard, with high performance communications to other hosts or display terminals via 100 Base T or better ethernet or optical fiber links. The system will employ a data acquisition subsystem based on a board developed by Rector at Los Alamos, that provides camera control, and high-performance multi-channel A/D. Although the technology is compatible with standard video technology, it is designed for high performance imaging with ultrasensitive, high frame rate, high dynamic range, and extremely compact imagers.

A strategic design decision was made early in our exploratory R&D effort. A number of sophisticated frame-grabbers had appeared on the market, featuring large frame stores and on-board digital signal processors (DSPs). This arrangement had the potential to allow real-time processing of the anticipated video data stream. However, the prices of these boards often exceeded the price of the host computer, and special software tools were required to program and debug the DSPs. Instead we opted for host computers incorporating a PCI bus (capable of video rate internal data transfer) and comparatively simple and inexpensive framegrabbers. The continuing leaps in microprocessor and memory performance have allowed the host computer to keep up with processing demands without introducing the additional cost and complexity of the programmable DSP framegrabbers. Deep reductions in the costs of motherboards and memory also have ratified this decision.

The microprocessor will consist of a Pentium series device with a clock speed of at least 200 MHz. The motherboard will have a PCI bus and expansion slots and will hold at least 64 Mbyte of SD-RAM. This technology has access times that are only 15-20% of conventional DRAM, and we have observed substantial speedup in applications involving high speed data streams or processing of large data arrays. The system will be useful as a general purpose computer providing capabilities for data acquisition, processing and display. However, for more demanding applications the system can be dedicated to required realtime functions, and other functions such data visualization, advanced analysis and interactive control can be off-loaded to another machine. For this application, we have chosen to work with the Linux operating system instead of Windows. Linux supports IP protocols, X-windows based displays, multiple process threads, sockets and all of the Unix tools for event driven computing. Because source code for the Linux is available, the kernel can be stripped down to its essential core.

The data acquisition system used in previous work employs a commercially available PCI bus framegrabber (Matrox Pulsar). This board uses host computer memory for frame storage. It provides an on-board VGA-compatible display adapter that was used to drive the LCD illuminator used in an early prototype. Although this board is a workable data acquisition solution, we have identified a number of limitations that will affect the performance that we eventually hope to achieve. The board designed by Rector addresses all of these concerns. It will provide a 12 bit ADC to fully exploit the dynamic range of modern CCDs. We will achieve at least 30 MHz sampling rates to fully exploit high speed CCDs by Dalsa and other manufacturers. This will allow us to approach our design objectives for confocal imaging at video rates. Our design can be expanded to accommodate up to 8 data streams if multi-port cameras are required for higher performance.

The use of a proprietary data acquisition system will provide considerable technical flexibility as well as market advantages. The system is designed to provide all of the necessary timing and control signals to drive non standard CCD imagers and to implement features such as line skipping, pixel binning, video gain and offset control, etc. The system will be adapted to allow direct control of scanned illumination. For LCD or DLP devices this will involve direct access to the RAM controlling the state of the SLM. For mechanical scanning systems the control signal will take the form of digital pulses (for stepping motor control) or analog signals related to the required translation or rotation. The integration of data acquisition and flexible control functions will allow a level of synchronization and performance beyond that achieved to date with generic hardware.

Image Reconstruction and Visualization:

The key to virtual aperture microscopy is in the algorithms used to reconstruct confocal images from a time series of subsampled images. Although we have considered a number of approaches, to date our principal method has been a simple algorithm employing peak finding and normalization, based on a reference image acquisition. We have extended this algorithm so that a reference is not required; for some applications, obtaining a suitable reference may be difficult or impossible. We are developing an algorithm that employs local convolution or "spot modeling" to implement a virtual aperture, facilitating the development of smart apertures that can outperform the fixed (or adjustable) physical apertures of conventional confocal microscopes.

The synthetic aperture employed by the Virtual Pinhole Microscope can be constructed by a variety of techniques of greater or less sophistication. In general, each pixel within the target composite image array is defined as a weighted linear combination over one or more elements of a single component image plane (i.e. one of the component images within the stack). Figure 8B illustrates a simple but effective method for deriving a reconstruction algorithm that in turn defines a synthetic aperture. This method employs a calibration image collected under the same optical conditions as the sample image (i.e. with equivalent slide, medium, coverslip etc.) but without an actual in-focus sample. A stack of these images are acquired, one for each separate pattern of illumination. For this method, the number and arrangement of illumination pixels should be set roughly equivalent to the number of pixels in the imager.

In this reconstruction procedure, the number of pixels in the target composite image is set equal to the number of pixels in each composite image (analogous to spinning disk confocal systems). For each pixel location in x and y in the target image, the maximum value of this pixel location through the entire stack of calibration images is determined. The stack (z) index and the maximum value of are recorded in two arrays (denoted as Index and Maxval in the figure). For each pixel a normalization factor (NF) is computed: NF=Constant/ Maxval. These values are stored in an array for future use. Each array has the same dimensions as the composite image array to be constructed. During reconstruction, the calibration image stack is replaced by a sample image stack. For each pixel location in x and y, the Index array is used to determine the stack index of the source pixel within the sample image stack of component images. This pixel is multiplied by the corresponding element of the NF array and the product is stored in the composite image array.

If each illumination pixel were precisely aligned relative to the corresponding image pixel, the synthetic aperture might be formed by taking the value of a single image pixel or the sum over a defined set of pixels. However, as illustrated in Figure 9, the problems introduced by discrete sampling of a continuous function will in general invalidate this strategy. The left hand panel of Figure 9A illustrates a pair of Gaussian intensity distributions (for example corresponding to diffraction limited point illumination) in continuous form (above) and as sampled by a

discrete detector array (below). The intensity profile sampled through each of these Gaussian-derived peaks is illustrated in the right hand panel of figure 9A. 2D plots corresponding to the continuous and discrete-sampled forms of the gaussian distributions are illustrated in Figure 9B. Note that if the value of the peak pixel in the sampled distributions were taken directly as the value of the corresponding target image pixels, the resulting reconstruction of the calibration image would contain significant intensity fluctuations due to discrete sampling. The normalization step eliminates this problem.

A second example illustrates an alternative method for image reconstruction. In this method, a calibration image is useful, but is not necessary. The number and arrangement of pixels in the target composite image is set equal to the number of illumination points sampled by the imager, across the entire stack of composite images. The resolution of the imager is chosen to allow adequate sampling of the point spread function. However, the density of the composite illumination array may significantly exceed the resolution of the imager. If the form of the point spread function is known, it is possible to detect sub-pixel shifts in the location of the illumination spot—a condition known as hyperacuity.

If a calibration image is available, local peaks in each of the calibration component images are identified, and a small region of surrounding pixels are identified as the source region. This region typically spans the point spread function of the corresponding illumination point. The intensity distribution across this region can be fit with an appropriate model function such as a Gaussian, allowing determination of the relevant parameters (e.g. center location, peak amplitude, half-width). Based on this model it is possible to compute the integral intensity of light that would be passed by an aperture centered on the peak of the underlying continuous intensity distribution. This aperture can be expressed as an array of coefficients, one for each element of the source region. The value of each pixel in the composite image array is computed as a linear combination over the corresponding source region. Each element in the source region is multiplied by its associated coefficient, and the resulting scaled elements are summed.

If a calibration image is not available, intensity fluctuations due to differential absorbance or reflection across an image may prevent reliable identification of all of the local peaks. However since the overall structure of the pattern of illumination is known, these locations can be predicted. In one method, the patterns of identified local peaks are fit with a grid composed of low-order polynomial splines. Such a grid can accommodate the warping of the regular illumination grid introduced by optical aberrations. Source regions are identified around each of the grid points whether or not a local intensity peak is discernible. The aperture coefficient array can then be constructed as described above. Additional constraints on the model may be useful. For example, the form of the grid within some local region should be consistent between different component images, and the form of the point spread function should be locally correlated even though it may vary across the image. We have developed two classes of reconstruction method: an efficient calculation for real-time implementation, and advanced high resolution algorithms requiring substantial processing off-line.

Suggested Reading

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