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**SINGLE MOLECULE DETECTION USING CHARGE-COUPLED DEVICE ARRAY
TECHNOLOGY**

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Abstract

An ultra sensitive technique for the detection of fluorescent species in a flowing stream has been developed. The extension of this technique to the detection of fluorescently tagged nucleotides will be a significant benefit to one of the novel approaches for DNA sequencing being developed at Los Alamos National Laboratories.

The detection scheme is based on a novel mode of operating a Charge-Coupled Device (CCD) which greatly enhances the discrimination between fluorescence from the analyte and the background Raman scattering from the solvent. Register shifts between rows in the CCD are synchronized with the sample flow velocity so that fluorescence from a single species is collected in a single moving charge packet occupying an area approaching that of a single pixel while the background is spread evenly among a large number of pixels. Our research has demonstrated that this technique is highly effective for the detection of fluorescently labelled latex microspheres. With additional development, we believe that this technique will achieve single molecule detection.

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Introduction

Over the course of this project, significant advances have been made toward realizing the goal of single molecule detection. Presently the system is capable of detecting the fluorescence emission from 100 nm fluorescent spheres with an excellent signal to noise ratio. A plot demonstrating the detection of ten 100 nm spheres is shown in figure 1. An enormous amount of

Figure 1. Plot of ten 100 nm fluorescent spheres

effort has been spent in configuring and evaluating the optical system required to accomplish the task of single molecule detection. From the last report, the optical arrangement has again been adjusted to increase collection efficiency while maintaining the critical spatial imaging integrity. The CCD camera system has also been modified to improve its flexibility in this application. This has included interfacing the camera to an image analysis package known as IRAF (Image Reduction and Analysis Facility), developed by NASA, provides state of the art tools for the manipulation, reduction, and analysis of image data. New masking techniques for the adaptation

of the CCDs have been developed which allow for a more flexible readout approach. Another area of improvement has been that of the flow system. The precision optical flow cell has been modified and a new solvent delivery system has been acquired to increase the stability of the hydro-dynamically focused stream and maintain the strict flow profile required for this project. These improvements have facilitated the advance to nanometer sized fluorescent particles and similar modifications should ultimately yield the desired goal of single molecule detection. We are diligently continuing to pursue this goal, as well as exploring the possibilities of exploiting the current state of the technology to other areas. Primarily, we envision adapting this detection scheme to both liquid chromatography and capillary electrophoresis. The current sensitivity of the technique will make it an ideal detector for these two applications.

Experimental

During the course of this project, the entire optical system shown in figure 2 has been developed. This experimental configuration includes a Coherent Inova 70 five Watt argon ion laser capable of being operated in either single line or multiline mode in the 457.9-528.7 nm band. The output of the laser is fed through a half wave plate and a polarizing beam splitter which rotates the plane of polarization of the laser from vertical to horizontal. This combination of elements can also be used for a very sensitive method of excitation power control. The laser emission next passes through two cylindrical lenses which function to shape the beam in the rectangular fashion desired for illuminating the flow cell. The laser then passes through the flow cell and into a beam dump. Fluorescence collection is accomplished via a series of lenses and filters. The collection lens is a precision Nikon CF brightfield objective. We interchange between two objectives. One is a 10X objective with a numerical aperture of 0.30 and a focal length of 17.5 mm. The other is a 4X with a numerical aperture of 0.13 and a focal length of 38.2 mm. These objectives are used at infinite conjugate ratio which provides collimated light at the filter stage. In previous reports we have used a holographic edge filter to reject the excitation light and a bandpass filter which coincides with the fluorescence maximum. Our experiments have shown

that the holographic edge filter introduces a significant amount of optical aberration into the system and the gain achieved from excitation band rejection is lost as a result of the reduction in image quality. For this reason the 550 nm bandpass filter is the only wavelength discriminator being used at this time. The filtered light is focused onto the CCD with an anti-reflection coated achromat (FL = 180 mm). The fluorescence emission is detected with a PM 516 CCD operated in time delay integration (TDI) mode.

A unique camera control system based upon a Sun Sparc II platform has been developed to operate the CCD. This state of the art electronics package is now fully functional, complete

Figure 2. Optical schematic.

with the appropriate software necessary to operate the CCD in the proposed modes; however, the level of effort necessary to realize the completion of this highly specialized and flexible CCD controller turned out to be more than we ever anticipated. Originally, we were informed by Photometrics Advanced Technology Division that all we would have to do was to copy a design that they were developing for NASA. Unfortunately, their development schedule fell behind, leaving us with the only option of proceeding with the development on our own. This setback significantly slowed our efforts.

A major aspect of this project has been the development of the sophisticated CCD camera controller necessary to accomodate the varied and unusual readout forms as well as several significantly different CCD formats. This camera controller, known as the Advanced Imaging System (AIS) is a variable read rate, high precision CCD camera system designed specifically for low noise image acquisition and precise, highly flexible CCD operation.

The recent explosion of CCD manufacturing has produced a wide diversity of CCD formats and operating modes. These include devices as large as 4K x 4K and as small as a single pixel, and device formats ranging from the standard square format to linear arrays to mutiple device mosaics. All of these devices may have one or multiple readout ports and may incorporate different types of readout amplifiers on a single chip. In addition, a host of flexible operating modes have also been developed (normal single port readout, mutiple port readout, antiblooming integration, time delay integration, and MPP inverted or non-inverted integration). The ability to successfully and easily adapt to all of these devices is a non-trivial task. In this project especially, we require the ability to easily modify the operation of the CCD as well as the flexibility to incorporate at least two different devices. This level of sophistication is beyond current CCD camera controllers; thus, this project demanded the creation of a novel and superior controller which could take advantage of the current technology in the CCD industry.

At the outset we envisioned a controller which could, by the replacement of a small printed-circuit board in the camera head, accept any CCD regardless of size or format. All operating parameters including clock voltages, timing, and reaout formats would be completely

software programmable. The system noise should be limited by the inherent characteristics of the CCD and not the controlling electronics. Finally the controller should have the ability to read data anywhere from 10 KHz to 1 MHz. To meet these goals a modular camera system was built which incorporated many state of the art electronics technologies that have become available in recent years.

The heart of the controller is a set of three mini electronics boards mounted on a custom backplane. The three boards are the central processing unit (CPU), the digital signal processor (DSP), and the temperature/shutter controller (TSC); each board can communicate with the others via the backplane and all can function independently. The CPU board contains a Motorola 68HC11 microprocessor with 32 KBytes of RAM and 32 KBytes of EEPROM.

The 68HC11 has a variety of on board sub-systems including a serial communications interface, a serial peripheral interface, an 8 channel 8 bit analog to digital conversion system, and a timer subsystem. The microprocessor was obtained with Max-FORTH (a high level modular computer language) already installed in ROM. The CPU card issues commands to the DSP and TSC cards; it can receive its instructions from either a serial terminal interface (at which the user can input FORTH commands directly) or from a host computer whose instructions it can interpret.

The DSP card is responsible for direct camera operation. It was selected for its very high speed operation (27 MHz) with very little overhead. Also included on the DSP board is 8 KBytes of memory, 16 bit latched input and output ports, and 5 MHz synchronous serial data interface. In the AIS camera the DSP is used to issue very specific base level commands to the Clock and Analog modules (to be described later) which operate the CCD. The simple subset of commands is designed such that any conceivable operation that one might require of a CCD can be performed via combinations of base commands. The DSP can operate at very high speeds since it only 'remembers' a very small number of these base commands (32 maximum) and it receives all of the necessary operating parameters from the CPU. To initiate a command to the camera the CPU needs only to write a command designator (between 1 and

32) to the command vector register of the DSP and from that point the DSP carries out the required functions on its own.

The TSC card is also controlled by the CPU. Its function is very simple, but requires high precision and reproducibility. As the name suggests the TSC card operates the shutter for the camera and controls the temperature of the CCD. The shutter operation is a simple on/off transistor arrangement and is controlled by TTL logic levels from the CPU. Temperature control of the CCD is accomplished in the following fashion. A specialized platinum resistor which has a specific, known change in resistance with temperature is attached to the base of the CCD. Applying a constant voltage to the resistor allows us to measure the current which is directly proportional to the temperature. The current is measured with a high precision instrumentation amplifier and read by the analog to digital converter module on the CPU board. The CPU intermittently checks the temperature of the CCD, compares it to the user's predefined desired value, and then turns adjusts a heating resistor if necessary (note that cooling is provided by the liquid nitrogen dewar which is attached to the CCD via an aluminum cold finger). This entire process occurs in the background such that normal camera operation is never interfered with.

As mentioned above the CCD is operated by the DSP which sends commands to the Clock and Analog modules. These two modules are large format printed circuit boards which are housed in a separate temperature regulated case attached directly to the camera head. The Clock board, as its name suggests, is responsible for the timing of all the signals that are fed into the CCD as well as the timing of the readout signals coming from the CCD. All of the clock timing and clock voltages are produced from signals obtained from the DSP which the Clock card interprets through a series of digital to analog converters, switches, and latches. The clocking waveforms are conditioned and shaped on the Analog board and then sent directly into the camera head. Inside the camera head there are two small boards which control some of the very simple tasks of the CCD's operation. One card is the interface the Analog module and the other is the socket card for the CCD. Since all signals enter the

camera head via the interface board. One only needs to change the socket card to change the CCD. This is a relatively simple process which can be accomplished without disturbing any of the other components of the system.

Data is collected and processed from the camera head by the Analog module which contains a dual slope integrator (which reads the charge stored in each pixel) and an extremely linear, high precision 16 bit analog to digital converter. The Analog module is responsible for collecting the analog data from the CCD, digitizing it, and sending it to the host computer over a serial interface.

This series of modules which comprises the AIS camera can be attached to any host computer which has a VME bus interface. In our configuration, the host computer is the Sun Sparc II workstation. The system is UNIX based and includes 32 Mbytes of RAM, two 207 Mbyte SCSI hard drives, a 150 Mbyte tape drive, and a 19" color monitor for user interface and image display. The Sparc is connected to the camera via a specially designed VME interface board which has its own microprocessor and 8 MBytes of memory (upgradeable to 32 Mbytes) for storing image data. The interface is a slave such that it cannot take control of the VME bus; it merely waits for a data stream which it writes to its own memory or a request from the host computer which reads out the memory. The memory is dual-ported so that these actions can be carried out simultaneously.

The software required to operate the various modules of the AIS camera is a rather complex but highly effective compilation of code. The CPU requires code written in FORTH that will operate all of the various camera modules. The CPU stores all of the current CCD's format parameters, the operating voltages and timing states, and the desired readout mode. The DSP is programmed in assembly language for high speed operation. It receives all of its commands and the necessary parameters from stored data tables which it obtains from the CPU. When the user initializes the camera, the DSP presets all of the operating parameters on the camera and waits for instructions. The user can then issue a command which the CPU processes and then passes to the DSP as the appropriate command vector. The bulk of the

software is the code written for the Sparc (in the C language). This software is responsible for interpreting user commands and passing them to the CPU as well as data acquisition and manipulation. The integration of the software and hardware in the AIS camera provides a sophisticated and flexible system capable of advanced, scientific CCD operation.

The AIS camera system accomplished the above stated goals extremely well. It is a fully programmable CCD camera controller compatible with any CCD. At slow read rates, the camera has demonstrated exceptional noise characteristics (< 5 electrons). In order to speed up development we chose to limit the upper read rate to 100 KHz. However, should a need arise in the future for high speed readout, a relatively minor modification to the the Analog card will provide the high speed capability. As was stated above the camera is capable of operating in full frame readout or time delay integration mode. It also provides us with the option of changing devices in the future. We have complete software control of the readout format which allows us to experiment with different masking arrangements and optimize these for different situations. Clocking waveforms can also be optimized to reduce noise and obtain the best possible TDI readout modes. Finally, the AIS camera has the ability to operate multiple cameras, multiple CCD's, or multiple ports on a single CCD. Thus, when the project progresses to the point of multi-wavelength detection we will possess the technology to incorporate a variety of detection schemes.

Our research has produced a camera capable of this unique TDI type of readout and investigations have shown that TDI mode imaging can be successfully implemented to track small fast moving targets in a relatively large background field. As an initial test a phosphor screen illuminated by an electron gun was used as a target; such a system produces a small, relatively bright, and rapidly moving target much like that in the actual experimental setup. Imaging was conducted in both normal and TDI modes. While normal readout at even the fastest shutter speeds produced a streak across the entire viewing region, TDI mode readout produced a highly localized spot with a much larger intensity. These results were very encouraging and proved that our original hypothesis was on track.

While modeling calculations based on the PMT results at Los Alamos demonstrate sufficiently high signal-to-noise to ensure the success of both single molecule detection and subsequent single molecule identification as well as indicate initial starting experimental conditions, a careful series of investigations has been conducted to achieve optimal conditions. The AIS experimental camera system which has been developed is particularly flexible allowing software selectable clock rates, "binning" parameters (21), and observation zones.

The final portion of this project will be to incorporate wavelength resolution into the detection in order to distinguish the four different color emissions corresponding to the different tags placed on the DNA nucleotides. The technology will be transferred to Los Alamos upon completion.

Background

The significant contributions to human welfare resulting from determining the entire nucleotide base sequence of the human genome are becoming more obvious almost daily. The human genome is the representation of the DNA in a person's body. It consists of approximately 3×10^9 nucleotide bases. There are four different bases in the human genome: Adenine, Thymine, Guanine, Cytosine. It is the precise sequence of these nucleotides in the genome which determines a person's past and future heritage. In addition, there are over 3,000 known genetic diseases which result from disorders in the base sequence. However, with current technologies, sequencing over a billion bases will be extraordinarily time consuming. The Keller-Los Alamos National Laboratory program has made significant progress toward realizing an instrument capable of sequencing a single fragment of DNA several tens of kilobases or more in length, at the rate of several hundred to a thousand or more bases per second (1). The basic concept involves labeling the nucleotides with highly fluorescent base specific tags, selecting a single desired DNA fragment, suspending the fragment in a flowing stream and sequentially cleaving labeled bases from the free end of the DNA fragment followed by fluorescence detection and identification in a

flow cell of the cleaved nucleotide. A diagram depicting this sequencing technique is shown in figure 3.

Figure 3. Sequencing schematic

Charge Coupled Device Detection in Flow Cytometry

Overview

Detecting and identifying the single nucleotides as described above is non-trivial. Success requires finding and identifying the fluorescently tagged species in a relatively large volume (on the molecular scale) of solvent molecules. The Keller-Los Alamos team has made highly significant inroads into this problem using hydrodynamically focused flow cell techniques and laser excitation (2). Their group has shown that for a Rhodamine-6G molecule passing through the detection volume (transit time 1 ms), 13.2 photoelectrons from molecular

fluorescence and 188 photoelectrons from background emission (presumed to be mostly Raman scattering) are observed (3). Thus, the signal-to-noise ratio (S/N) for single molecule detection is $S/N = 13.2 / \sqrt{188 + 13.2} \approx 1$. Techniques for discrimination between background emission and fluorescence to improve the signal-to-noise are under study. Keller's team is currently investigating the use of a microchannel plate position sensitive detector with time correlation of suspected photon bursts. Data processing is done with software on a Sun Workstation. The results are encouraging (4) and this approach is being actively pursued.

Fundamentally, the bottom line with the signal-to-noise problem is that Raman emission from the solvent (water) molecules occurs at the same emission wavelength as the tagged molecules. While the tag species can be cycled through emission $\approx 1 \times 10^5 - 1 \times 10^7$ times (5,6), there are many more solvent molecules in the flow cell so that the weak Raman emission vastly overpowers the fluorescence from the single tag molecule. Additionally, viewing of the excited region of the flow cell precludes the identification of more than one fluorescing species in the observation region at the same time. Proper use of a charge coupled device (CCD) holds great potential for solving both of these very difficult problems.

Scientific charge coupled devices are currently revolutionizing numerous areas of low light level spectroscopy (7-16). The use of a CCD detector represents the potential for a tremendous increase in signal-to-noise for single molecule detection. In comparison, using a single, non-imaging photo-multiplier tube for single molecule detection is like trying to determine the presence of a small moving bright spot in a large light background by looking at the difference in intensity of the total background with and without the bright spot. The CCD will let us concentrate on a small area containing the bright spot without prior knowledge of where the bright spot will appear and continue collecting photons into a growing charge packet from this spot as it moves along the flow cell. Feasibility calculations demonstrate that the background problem is greatly reduced and single molecules should be detected easily with excellent signal-to-noise. These devices, when properly manufactured and operated, show improved quantum efficiency (QE), lower dark currents (on the order of 8×10^{-4} electrons per pixel per second) and

the ability to enhance signal-to-noise ratios through combining the multiplex advantage of a large number of detector elements or pixels with a variety of novel readout modes when compared to other detector technologies. Further-more, we envision the commercial DNA sequencer of the future, based upon our approach, will use a diode laser for the excitation source and near-infrared emitting dyes as tags. A CCD with excellent quantum efficiency for detection of radiation in the near-infrared region is ideal for this application.

CCD Readout Modes

A unique mode of operating CCDs, first applied for aerial platform surveillance by the military (i.e., ground observation from satellites and aircraft), is an ideal solution for detecting one or more relatively "bright" sources in a large background field (17,18). Proper understanding of this mode of operation and the additional "twists" needed to solve the single molecule detection problem require some basic understanding of CCD operation. In all cases, we will deal with frame transfer devices (vs. interline).

A. In normal TV camera operation (19), half of the frame transfer device is blocked or masked and the picture image is exposed to the open region.

FIGURE 4A

B. Readout is accomplished by shifting the image rapidly in a parallel fashion under the mask (down).

FIGURE 4B

C. While the next image is being exposed, the parallel clocks on the masked region are clocked one cycle shifting the bottom row into the serial register C_1 and the entire serial register is clocked out, C_2 .

FIGURE 4C1

FIGURE 4C2

D. The lower image is clocked one row at a time followed by clocking and reading the entire serial register until the entire masked image has been read out in a serial raster format. While this occurs, the upper (exposed) image continues to integrate.

E. The next image in the exposed region is rapidly shifted via the parallel registers under the now empty masked region.

This technique assumes that no significant amount of light will fall on the exposed region during the high speed shift of the parallel registers. Photons arriving during the shift will contribute toward a smeared image. This scheme tends to work well at normal commercial video rates due to the frame speed and low dynamic range of the video image.

Time Delay Integration

The aerial platform surveillance technique serendipitously combines the fact that the image on the array detector moves as the platform moves in the air. A fully unmasked CCD is precisely aligned such that the parallel registers lie in the direction of motion and the parallel registers are clocked at precisely the rate the image is moving on the array. Every single point on the ground based image can be integrated into a moving pixel-sized charge packet from the time it first moves onto the array until that point leaves the array. This technique (17,18) generally termed Time Delay Integration (TDI), provides the ability to see faint objects at low light levels from a moving base with freedom from having any dead time associated with shutters or the transfer of the scene under the mask. Observation at low light levels is greatly enhanced since much longer integration periods are allowed without smearing effects. A similar approach is currently being explored for flow cell cytometry (20).

This same readout mode offers a powerful new capability for solving single molecule detection. If the emission of a laser illuminated hydrodynamically focused flow cell is properly imaged on the CCD with the flow channel aligned with the parallel axis and the CCD is clocked at a rate synchronized with the image of the central flow, the fluorescent emission from the tagged molecule will form a charge packet moving with the molecule, integrating charge from additional

fluorescent photons as the molecule travels down the flow cell. With the proper geometry, optics and controls, this proposal will demonstrate that the packet can be held, in a worst case situation, to a region corresponding to a few detector element pixels and can approach total confinement in

Figure 5. Pictorial representation of TDI imaging

an area equal to one or two pixels.

The significance is that, under these conditions, the Raman signal from only the volume integrated into these small area charge packets is added to the fluorescent signal rather than the Raman signal from the entire cell. Dependent on image magnification and the CCD employed, this factor alone results in approximately a 200 to several thousand fold discrimination against the Raman background. As this proposal will demonstrate, this improvement, coupled with the improved quantum efficiency of a properly configured anti-reflection coated CCD at the intended observation wavelengths, can be expected to give an easily observable signal-to-noise for a single tagged molecule. Presence of a charge packet from a tagged molecule will be evident through sequentially reading individual pixels or small groups of pixels, so called binning, see Epperson, et al (21).

Additionally, this approach allows a number of tagged molecules to be in the flow cell simultaneously as long as they are separated vertically by several pixels. Therefore, between

twenty and several hundred molecules may be present in the observation region at once and observation rates of several hundred to many thousand tagged molecules per second may be observed.