In vivo and In situ Cellular Image Processing and Characterization: Challenges and Solutions

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Abstract: - We study the feasibility of 3D virtual histology through in vivo and in situ cellular imaging. A prototype system has been developed based on photodynamic fluorescence signals, confocal endomicroscopy, and FPGA image processing and characterization computing. Experiments in its clinical applications have been conducted, mainly for diagnosis of early-stage mucous cancer. With the fine-grained parallel imaging programs mapped on the FPGA, a stream of focused optical sections of microstructures in the subsurface layers up to 300µm in depth, can be processed online and the extracted features can be visualized seamlessly with the endomicroscopy settings.

Key-Words: - Cellular imaging, photodynamic fluorescence, endomicroscopy, FPGA, fine-grained parallel computing, virtual histology

1 Introduction
We study the feasibility of 3-dimensional virtual histology which enables non-invasive high-resolution magnified imaging at cell level. Such an imaging modality is beneficiary as no extraction by biopsy is required. This reduces the risk of bleeding, infection, perforation or mechanical agitation that can lead to the spread of tumor cells through blood and lymphatic vessels [1] and the possible complication that might occur in the invasive biopsy procedure. Moreover, the imaging is carried out in vivo which is crucial as the stroma and microenvironment of the tumor influences the development progression of the tumor greatly [2]. In addition, being non-invasive will remove one possible source of confounder, and it will allow future extension of using the same modality in clinic settings instead of the current research settings.

Cellular imaging, through the use of molecular imaging probes allows targeted morphological imaging. The molecular imaging probes are specially designed to target specific biomarkers with high affinity, allowing the correlation of imaging signal strength to molecular signatures. These biomarker-correlated images further provide histological confirmation, allowing the staging and grading of the virtual cells samples based on the morphological details in the image.

The optical fluorescence imaging is an imaging modality that consolidated various crucial techniques including the confocal, endomicroscopy and fluorescence technologies. It fulfills in vivo imaging at subsurface depth level and with sub-cellular microscopic resolution. Moreover, there are a number of near-infrared fluorochromes and activated fluorescent proteins [3] based on green fluorescent protein, luciferin, and photosensitizers [4] that emit light energy at a different characteristic wavelength from the absorption wavelength. Photosensitizers have been identified within the different types of imaging probe as the most suitable probe. It has been observed that photosensitizers do specifically accumulate in neoplasm and cancerous cells, allowing the correlation of molecular basis of targeting to the morphological details.

The in vivo cellular imaging reflects the cellular and molecular processes in living subjects. As such, in order to monitor and analyze the traffic of the imaging probe, an interactive image processing and analysis computation system is necessary. The image processing requires a computing system that allows fine-grained parallel computation for sophisticated analytical algorithms. Field Programmable Gate Array (FPGA) is an emerging technology that allows reconfigurable parallel processing at hardware logic level [5]. The synthesis of the algorithms as hardware logic circuits onto the FPGA chip allows the implementation of embedded image processing and analysis.

We have been developing a prototype system [6] based on the photodynamic fluorescence signals, confocal endomicroscopy, and FPGA image...
processing and characterization computing. The challenges are twofold. Firstly, each of the 2D arbitrarily taken images slices suffers from possible movement and tissue expansion or contraction. This means that the exact coordinates misalignment and geometrical distortion of the 2D image slices is unknown. The reconstruction of 3D spatial information from these 2D misaligned and distorted image slices, is an immense difficulty. Secondly, beyond a certain time frame, the 3D volumetric images will be different due to physiological changes. Thus, the deciding factor of whether to reconstruct certain slices into the 4th dimension (temporal) as oppose to using it to refine the existing 3D spatial volume needs to be identified and considered. The slow rate of image acquisition and low signal to noise ratio (SNR) due to low photon count in fluorescence imaging complicated the matter even further.

2 A Virtual Histology Protocol

In a conventional image-based cancer diagnosis method, suspicious tissue to be investigated is extracted through a surgical procedure called biopsy [1]. One of the two types of biopsy, the incisional biopsy or the excisional biopsy will be performed. The incisional biopsy will remove a sample of tissue, while the excisional biopsy will remove an entire lump or a large volume of the suspicious tissue.

The large volume of tissue is sliced by a microtome into thin segments for mounting onto microscope slides. In the case where the tissue sample obtained is sufficiently thin, it is mounted directly onto microscope slides without slicing.

Before the microscope slides are covered with the cover slip, the tissue samples are stained with dyes such as the widely used hematoxylin and eosin (H&E) stain. The prepared microscope slide containing the tissue sample is then viewed under the microscope. A charge-coupled device (CCD) is used to capture the images seen through the microscope.

A stack of 2D images representing the 3D volume is subsequently analyzed by histopathologist. Occasionally, the images to be analyzed by the histopathologist are pre-processed offline to yield enhanced images.

This traditional cancer diagnosis workflow has been widely employed by the clinicians, despite being associated with a number of major problems. Biopsy tissue extraction, especially the excisional biopsy is an invasive procedure. There may be complications from anesthesia and the biopsies may be non-representative due to the heterogeneity of cells in a single cancer site and the nature of sampling, leading to the underestimation of the severity of the cancer and the tumor invasion. Moreover, the decision to obtain the sample is based on the white light visualization of the tissue, which poses the inability to differentiate flat malignant lesions from normal or inflamed tissue [7, 8]. In addition, the chemical used to preserve the tissue sample causes cell shrinkage, and the slicing of the tissue cause distortion.

To solve the above problems, we have explored an in vivo cellular imaging protocol, thanks to rapid development of new molecular probes and microscopic imaging instruments. A photodynamics-based approach is proposed for a virtual biopsy protocol.

![Figure 1: The proposed workflow of virtual histology](image)

As outlined in Figure 1 above, the tissue is instilled with a solution consisting of photosensitizer and enhancer. The photosensitizer can act as a molecular imaging probe while the enhancer can improve the solution’s ability to penetrate tissue.

An endomicroscopic probe is inserted through cavities such as nasal cavity or oral cavity to the tissue volume under investigation. Spectrum band-limited laser light of the photosensitizer’s absorption range is delivered using confocal technology to the tissue through optic fibers within the probe. The emitted fluorescence light from the tissue is captured using a photo-multiplier tube (PMT). An image volume is obtained by the continuous scanning (optical slicing) through the volume.
The acquired image volume is analyzed and rendered using sophisticated computing technologies such as a field programmable gate array (FPGA) embedded computing system, with our fine-grained parallel imaging algorithms.

3 Fluorescence Signals for In Vivo Cellular Imaging

The molecular basis of our in vivo cellular imaging is photodynamics in which a target-specific molecular imaging probe, or photosensitizer, undergoes photoactivation. The photosensitizer compound absorbs electromagnetic radiation, becomes energized to a higher energy state, and releases the energy into chemical energy, light energy and heat. The light energy is in the form of fluorescence light emission.

There are two imaging probe strategies, namely the direct and indirect strategies [9]. Direct imaging probe strategy images the target directly using a target-specific probe, resulting in imaging the probe location and intensity that is related to the quantity of the target and affinity of the probe to the target. The limitation of this strategy is that the quantity of the target may not be related to the activity of the target, as the activity of the target depends on numerous factors. The indirect imaging probe strategy acts in a manner where the imaging probe reacts to a molecular component, that interacts directly or after a cascade of interaction with the interrogatable label component that is being imaged. A drawback of this method is that the intermediates may create confounding factors.

Use of photosensitizers provides molecular targeted imaging, which is an advantage over the conventional imaging stains. The commonly used direct imaging probes for nuclear imaging includes monoclonal antibody targeting of a particular cell membrane epitope, such as an enzyme using an enzyme-specific antibody probe or a transporter using a transport-specific antibody. In addition to antibody, the probes can be minibodies, peptides, antisense oligonucleotide and aptamer oligonucleotide [9, 10]. A common indirect imaging probe is based on the reporter gene and probe pair. In reporter gene method, the reporter gene product, which can be an enzyme, converts a reporter probe to a metabolite that is selectively trapped and imaged [9, 10].

An interesting category for optical imaging is the fluorescence probes. They can be designed to employ either strategy. Fluorescence probes are those, which emit a different wavelength in visible light energy from that they absorb. Examples include the use of fluorescence proteins such as green fluorescence protein (GFP), the red fluorescence protein (RFP) [11, 12], and the use of photosensitizers [13, 14] such as 5-aminolevulinic acid (5-ALA) or 5-ALA esters.

It is noted that photosensitizers by itself, when rinsed over the live mucous cells, is taken in at an extremely low rate. The mucous epithelia cells are always covered with a layer of dead cells, proteins, and mucous. This barrier layer prevents the photosensitizer from reaching the live mucous cells. Thus, image of extremely bad quality can be obtained when it is imaged directly. As such, the photosensitizer is formulated along with an enhancer that aids the photosensitizer in its penetration of this barrier.

4 Embedded Computing for Image Processing

The cellular imaging tasks defined in our approach demands a fusion of several instrumentation technologies. A processing unit is connected to the probe by means of optic fiber and electrical cables. It consists of a dichromatic mirror, a low-power laser generation unit, a photomultiplier tube, and a computation unit.

Celoxica RC300 [16] is a reconfigurable device, with a Virtex-II 2V6000 FPGA chip [17]. The single-chip device contains programmable logic and programmable routing resources. The device is configured with the logic and routing resources taking a particular state, allowing the design to be implemented in a fine-grained parallel and optimized manner.

We used the hardware descriptive languages Handel-C [18] and Verilog [19] for designing the programs. Celoxica DK and Xilinx ISE are used as our primary developmental environments. Taking advantage of the simulation ability of Celoxica DK developmental environment, the clock cycle, the threads running in parallel and the values of the variables taken are monitored and analyzed.

This ability to monitor the threads that are running in parallel with the variables’ values along with the clock values, allows us to verify and debug the changes to the variables’ value in the midst of multiple parallel threads, the parallel and sequential relations of the numerous threads, and the amount of clock cycles required for each operation. Thus, the simulation results in the verification and debugging of the algorithmic accuracy of the Handel-C design, and its usage in
conjunction with re-designing the non-optimized portions identified, results in the optimization of performance efficiency.

The Handel-C design is subsequently compiled to lower-level hardware description languages (HDLs), such as EDIF, VHDL and Verilog. Though the compiled design is optimally performance efficient and algorithmically accurate, it is not resource optimized. Thus, we further resource optimized this performance optimized design that has been compiled to Verilog in the Xilinx ISE environment. We view the synthesized Verilog logic block in Xilinx ISE environment as shown in Figure 4, and in conjunction with re-designing the Verilog code, the design is being debugged and optimized at the lower resource level.

In addition, the performance and resource optimized logic level circuit is again simulated using ModelSim along with the resources’ timing constrain values. The input ports are provided with a set of phantom data, and the values taken by each of the variables are monitored, through the waveform diagram provided by ModelSim. The input and output values that are tested using the ModelSim test-bench are compared with the same design in Matlab using the same test-bench values. This comparison provides evidence that the hardware implementation, which takes into consideration of resources’ timing constraints still provide the same results as formulated by the algorithms.

The image processing algorithms thus can be implemented in the following major steps:

1. We design each of the algorithms in Handel-C, refine the code for parallel processing of the pixels with minimal clock cycles, and further optimize the logic block arrangement and pipelining through the use of registers in Verilog code.
2. Following, the logic of the design is synthesized. This logic design is translated to the gate level design. This gate design is mapped to the gate primitives available in the target FPGA chip.
3. Subsequently, these assigned gate primitives are placed onto the physical gate positions on the chip. The wires are routed to link the gates and the FPGA configuration instructions consisting of the physical layout are generated.
4. These FPGA configuration instructions are then loaded onto the FPGA chip.

The Virtex-II 2V6000 FPGA chip used in the implementation contains six millions logic gates that are arranged as array logic elements. Each of these logic elements provides the configurable system gates that are used to synthesize the combinatorial and synchronous logic and as basic storage elements. As such, in the designing of the algorithms, full use of parallelism is of focus, in order to obtain the highest possible design speed for the algorithm. This is the crucial factor in the generation of image processing algorithms that fulfill the criteria of in situ image processing.

In a typical scenario, the object of interest is 3D and colorful. In order to process the images obtained from the object, to visualize the object in various views, and to enable the process and visualization results to be computed in situ, substantial amount of computation cycles will be required. The amount of computation cycle is tremendous. Even at the slowest rate and resolution of digitized PAL analog input, images are generated at a rate of 25 full frames per second with 768 by 576 pixels. As color images have three components, there are around 33 million inputs per second to be processed and rendered for visualization. Thus, any 3D vector image processing algorithm will require such substantial computation power that a serial-instruction based computer will not be able to process in time. The FPGA design of the algorithms takes full advantage of the parallelism available due to its chip-level architecture.

A full working prototype has been developed with the capability to process analog input video signal at 25 full fps PAL or 30 full fps NTSC with various image processing filters that can be applied concurrently. In addition, the prototype is equipped with a touch screen user interface that interactively allows the user to apply various functions and filters with a simple touch on the screen. It also features an informative panel that displays the histograms and other measurements.

We classified sixteen menu buttons into four main categories consisting of video setup functions, image processing algorithms, image measurements functions and image magnification functions. The image processing algorithms are further categorized into arithmetic operation filter (invert color filter), neighborhood operation filter (median de-noising filter), convolution operation filter (smoothing filter, sharpening filter and Sobel edge detection filter), segmentation filter (threshold outline filter) and frame difference filter (motion detection filter). In addition, we provide a function to freeze and unfreeze the current video frame, such that the image of interest can be
The image measurement functions provide the histograms information and the minimum, maximum, median and mode measurement values. These values are provided for each of the red, green and blue color channels, with the selection area being either the entire image or the selected region(s).

The image magnification functions are used to interactively magnify, de-magnify or reset the video input area to be viewed and processed. This action of digital magnification is interactively reflected on the selection area for image processing and measurement, which is simultaneously being updated accordingly.

Discussion and Conclusion
We have been working on an in vivo and in situ navigational cellular imaging system, with feedback and dynamic spatial, contrast and temporal optimization for confocal endomicroscopy. A new clinical concept of virtual histology has been introduced. A stream of focused optical sections of cells and tissue structures in the subsurface layers up to 300µm in depth, can be processed online and visualized seamlessly with the endomicroscopy settings.

The bi-directional interface provides a means for the captured video stream to be directly transmitted to the FPGA computing system and for the FPGA computing system to keep track of the spatial coordinated of the image acquisition as it transmits commands to the fluorescence confocal endomicroscope, mimicking the human controlled acquisition of the images.

The sophisticated algorithms used in the computation include image processing, analysis and visualization, for both grayscale and multiple-spectrum images. The algorithms are implemented at circuit level with fine-grained parallel computing. This implementation process involves the re-designing of the algorithm for FPGA parallel hardware architecture, the logic synthesis of the design, the translation of the logic design to gate level design, the mapping of the gate design to the gate primitives available in the target FPGA chip, the assignment of gate primitives available in the FPGA chip to actual physical gate positions on the chip (placement), the connection of the wires linking the gates (routing), and the generation of the FPGA configuration instruction consisting of the physical layout.

The work will be a gigantic move that translates the research based system into a clinical based system. Meanwhile, numerous safety and regulation issues will need to be addressed. Tremendous efforts will be required to fulfill the objectives of this in vivo and in situ navigational cellular imaging system, so that the development of the system will be successful, which can provide profound health benefits to the society. It will enable further advancement in the field of basic cell biology, aid our understanding of the mechanism of disease progression and allow the monitoring of drug effects at the cellular level.

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