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OP-15

HOLOGRAPHIC IMAGING OF CELL PROLIFERATION

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INTRODUCTION: In this study we aim to demonstrate the use of holographic imaging as a method for observing biological processes as a means to gain a better understanding through the depth information of the sample.

METHOD: To this end we conducted an experiment where the proliferation of ONCO-DG1 cells were observed over a 48-hour period using a Mach-Zehnder interferometer. A total of eight samples with initial cell counts between 2000-10000 were observed and the total cell counts were reported for 6, 12, 24 and 48-hour periods. We used a modified watershed segmentation technique for segmenting and counting individual cells.

MATERIAL: We compared the cell counts obtained from the holographic images with cell counts obtained via capacitive cell cytometry methods from similar samples. We observed that the holographic cytometry method achieves high sensitivity and specificity against manually annotated samples. Moreover, holographic method gives a depth information which could be used to assess the growth pattern of each individual cell in addition to the proliferation of the whole sample.

RESULT: The results indicate that the holographic imaging method presents clear advantages over standard light microscopy techniques for assessing the proliferation and growth of 2D cell cultures. Through holographic imaging methods we can measure the localized proliferation patterns and single cell growth pattern for better understanding the dynamics of the proliferation process.

Keywords: holographic imaging cell proliferation

INTRODUCTION

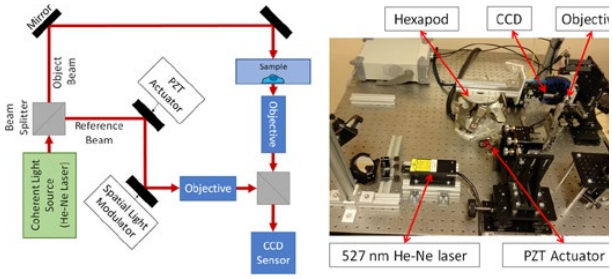
Imaging of biological specimens by means of classical optical techniques presents challenges due to their highly transparent nature [1]. Interferometric techniques extract the phase information from the observed sample to obtain quantitative depth measurements [2]. In particular digital holographic microscopy techniques have been gaining popularity due to its high accuracy and non-destructive nature. Use of DHM for imaging of live cells has been demonstrated through numerous application such as analysis of human hepatocytes [3] or observation of mouse cortical neurons [2].

In this study, we investigate the use of DHM for observing the cell proliferation patterns of in-vitro tumor cells. In particular we culture 8 samples with 1000 through 8000 ONCO-DG1 cells inside 480µL micro-wells and holographically reconstruct the well in 6-hour intervals. Using the acquired depth maps, we report the results for our noise reduction, cell segmentation and cell counting algorithms.

MATERIALS AND METHODS

Sample Preparation

We used 480µL micro-wells for culturing each of the samples. The wells were fabricated by replica molding from an SU-8 (MicroChem)/silicon master based on a ratio of 10:1 to curing agent. The master mold was produced by ultraviolet (UV) lithography. After pouring PDMS on a mold, the mixture was degassed in a vacuum chamber for air bubble removal and cured at 80°C for 1 hour in the oven. The devices were then cut out by a razor blade; the fluidic connection ports were punched, and bonding to a glass slide was done after oxygen plasma of both surfaces and conformal contact (Harrick Plasma cleaner/sterilizer, 4 mbar, 120 s).



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Figure 1: Schematic diagram of the setup and (b) its experimental counterpart.

Holographic Imaging Technique

For almost all cell manipulation tasks, the morphological structure of the manipulated cells is a critical parameter. Cell morphology is an important determinant of the cell genotype and determines many of its characteristics including its surface adhesion behavior its intra cellular signaling pathway regulation and its membrane permeability. It is also a stronger indicator of the class a cell belongs to, compared to classical light microscopy techniques [4]. We investigate the use of morphological information in cell transportation operations. Assessment of the cell shape and cell-surface adhesion helps better understand how to direct the actions of the manipulator and how to lift and carry the without compromising its integrity.

To obtain depth maps from the sample we use a phase shifting in-line Mach-Zehnder interferometer, as shown in Figure 1, in which the phase shifting is achieved by a high-frequency piezo actuator. Mach-Zehnder interferometry is based on the interference of a object wave which contains the wavefield transmitted from the observed sample and a reference wave which contains the wavefield of the original coherent light source. The coherent light source used in this study is a 527 nm He-Ne laser. The interferogram obtained as such is recorded by a CCD sensor. Using the complete set of interferograms obtained in equal intervals throughout a whole period of the wavefield is used to solve for the phase of the observed sample. This information in turn allows us to compute the depth information. The intensity distribution of a given interferogram can be written as

$$L_j(x,y)=A_o^2(x,y)+A_r^2+2A_o(x,y)\,A_r\cos[\theta_o(x,y)-\delta]$$

Where A_o and A_r are the object and reference wavefields respectively. The phase information of the sample can be reconstructed by translating the reference wave and solving the set of linear equations that form taking the Fresnel transformation of the complex wavefield that is recorded via an image sensor. In this study we follow this approach using a PZT actuator for shifting the phase of the reference wave.

Holographic Data Processing

Image Stitching: Since the field of view of an imaging system (including ours) is rarely large enough to view the whole sample, we developed a depth based stitching algorithm that composes a depth map of the whole sample by combining together smaller patches. To this end we coupled the sample holder to a 6-axis Hexapod and moved the sample in the XY axis in a stepwise fashion while recording the interferogram at each step. The depth maps, reconstructed from these interferograms are then stitched together using the method described in [5].

Cell Segmentation: After acquiring a depth map for the whole sample, we used a marker-controlled Watershed transform based segmentation algorithm [6] to segment the parts of the map that contain a single cell or a cell group.

Cell Counting: For each segmented section, we use a volumetric prediction method to get an approximate count of the cells within each region. The cell counting algorithm is based on calculating the mean volume of singular cells by clustering the lower end of the segmented volume values and dividing the volume of each segment with this base value. This gives us an approximation to the cell count inside that region.

RESULTS

We prepared four different samples with a known morphology for the assessment of our noise reduction algorithm, using micro spheres with 100μm diameter. We use the known morphology as ground truth and calculate the SNR values for raw and processed maps as follows,

$$SNR = 10 \cdot \left[\frac{\sum_0^{n_z-1}\sum_0^{n_y-1}[r(x,y)]^2}{\sum_0^{n_x-1}\sum_0^{n_y-1}[r(x,y)-t(x,y)]^2} \right]$$

where $r(x,y)$ is the reference depth map and $t(x,y)$ is the noisy depth map. SNR values are compared for, four different images are compared for different σ values are compared in Table 1. The reconstruction obtained from Sample 1 and Sample 2 at 0 and 48-hour marks are given in Figure 2.

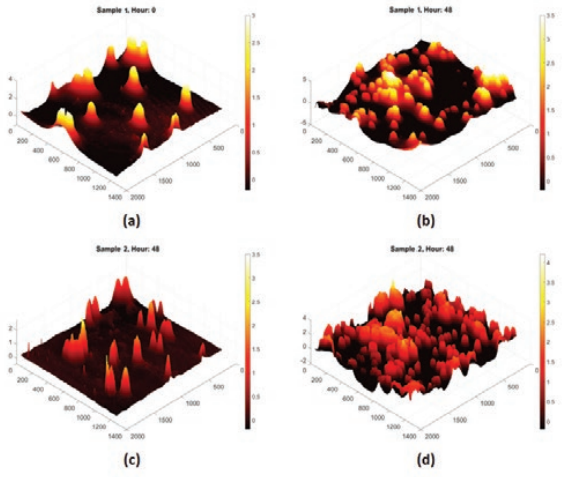


Figure 2: Holographic reconstructions of some example samples. (a) Sample 1 at its initial condition and (b) after 48 hours. (c) Sample 2 at its initial condition and (d) after 48 hours.

For the cell segmentation algorithm, we used manually annotated segmentations as ground truth and compared them with automatically segmented regions using the intersection over union, sensitivity and specificity metrics which are given in Table 2. The method used for calculating these metrics is as

$$Sensitivity = \frac{TP}{TP+FN}, \qquad Specificity = \frac{TN}{TN+FP}$$

where *TP, TN, FP* and *FN* refer to true positive, true negative, false positive and false negative respectively.

	Reference 1	Reference 2	Reference 3	Reference 4
Raw SNR	29.95	28.25	29.21	22.49
Filtered SNR	37.07	36.82	37.82	35.25

Table 1: SNR values of raw and filtered depth maps for reference samples.

	Sample 1 @ 0h	Sample 1 @ 48h	Sample 2 @ 0h	Sample 2 @ 48h
Sensitivity	90.27%	87.45%	90.89%	89.12%
Specificity	91.08%	88.67%	92.03%	91.02%

Table 2: Sensitivity and specificity values for two samples at 0 and 48-hour marks.

CONCLUSIONS

In this study we investigated the use of digital holographic microscopy method for cell cytometry. In particular we developed some depth processing algorithms which could be used for cell segmentation and cell counting. We also reported the performance of these algorithms against manually annotated samples. The results indicate that the DHM method achieves high sensitivity and specificity for the cell segmentation task. Also, we show that the volumetric information obtained from the depth map of the cell culture can be used to acquire a more accurate cell count prediction for regions in which individual cells can't be segmented It is clear that the DHM method will play a critical role in the future for cell culture studies where the morphological structure of the sample is important.

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OP-16

DISCOVERY OF METHYLATION BASED IMMUNE-ESCAPE MECHANISMS IN RARE CANCERS

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INTRODUCTION: Tumors should escape from immune system to survive. Studies showed that DNA methylation has role in negative regulation of immune system (a.k.a immune-escape) which can be controlled with demethylation agents. Patients with rare cancers suffered from low number of drugs since for most of them drug development is non-trivial and non-profitable. Enlightment of methylation based immune-escape mechanisms are important since regulation of DNA methylation is possible with approved drugs. Aim of this study is creating a bioinformatics pipeline and apply it to ten rare cancer type to reveal common and type specific immune-escape mechanisims rooted from abnormal methylation patterns.
MATERIAL&METHODS: The developed pipeline starts with gathering data from TCGA and normalization. After that, differentialy methylated and expressed genes inferred. Candidate genes clustered according to their semantic similarity for enrichment and network perturbation analysis. Finally, statistically significant pathways and functions analysed and filtered manually based on literature.
RESULTS: In this study, type specific and common genes and pathways revealed for rare and reported with statistical and literature based evidence which might be used for new targets to therapies.
CONCLUSIONS: We discussed that new bioinformatics pipelines are important for rare cancers since known efforts are mostly focused to prevalent cancers. In this study we propose a new end-to-end bioinformatics pipeline and validate its efficiency by using literature results.
Keywords: Cancer, Methylation, Immune-escape

INTRODUCTION

Negative regulation of immune-system (immune-escape) is one of the key characteristics of the tumors [1]. This trait of the tumors also decrease success rate of the immunotherapy which is one of the most promising solutions to treat cancer. Hence, in the last decade, immune-escape has been one of the most studied phenomena for the cancer treatment. Negative regulation of the immune-system can be triggered through genetic or epigenetic alterations [2,3]. From an evolutionary point of view, epigenetic alterations are more common than the genetic alterations in the tumors since there are many mutation control and repair mechanisms are available in the cell [4]. Rare cancers are important since many people suffer from them. However, research and funding aims rare cancers are significantly lower than the common tumor types [5]. Since, funding bodies calculates return on investment numbers, it might be understandable but this does not change the fact that 4,400,000 people diagnosed with rare cancers only in EU [6].

If epigenetic roots of the immune-escape could be discovered these epigenetic changes might be reversed using known epigenetic regulatory drugs [7]. In this study we created a methylation analysis pipeline and investigate the abnormal epigenetic patterns which are supported by the differential expression patterns in ten rare cancer types.

The pipeline we created is an end-to-end workflow starts from the data download continues with the data preprocessing, differential methylation and expression analysis, calculating gene similarity according to the known immune-escape proteins and cluster them to increase specificity of the enrichment and pathway perturbation analysis. We then investigate on each gene and pathway in the literature to discover their relation with possible immune-escape activity.

MATERIALS AND METHODS

We use data from TCGA project and download it from the Genomic Data Commons (GDC) Data Portal. Methylation analysis data is produced by the "Infinium HumanMethylation450 BeadChip" covers gene regions with sites in the transcription start sites (TSS) , 5'UTR, first exon, gene body, and 3'UTR. In transcriptome profiling the downloaded data was preporcessed by the GDC according to "HTSeq - FPKM-UQ" workflow that can be summarized as "Quality Filtering", "Fragment Count", "Count Normalization" and "Upper Quartile Normalization" steps.

After this step the differentially methylated probes discovered. We only aim the distal probes (probes at maximum 2kb distance of the TSS) because we would like to discover down/up regulation of the proteins through binding of transcription factors at TSS which depends DNA methylation patterns. Since the number of the normal samples are not equal to the tumor samples in TCGA and there is no grouping is done based on tumor subtype, we choose unsupervised method for differential methylation analysis. In this method, DNA methylation beta values of the tumor and the normal samples ranked and the lowest %20 bound is selected for both types on each probe for the hypomethylated probes (highest %20 is selected). Afterwards, these values are tested using the one tailed t-test to see if there is a significant difference. This method could discover differences between tumors and normal samples even if the tumor can be composed by different subtypes.

To create a relationship between genes and the differentially methylated probes nearest 10 upstream and 10 downstream genes selected for the testing. Inverse relation between genes and probes are tested with Mann Whitney U test and the multiple test correction is applied using a permutation test. For the permutation based test a number of random probes selected and their p-values counted to find what percentage of these random probes could generate equal or lower p-value. This analysis serves for finding differentially methylated genes which methylation levels are significantly correlated with their gene expression levels. The empirical p-values are calculated in this step and introduced in the results. These genes are saved and used in further steps but we also use them to enlighten methylation based immune-escape mechanisms through literature search. ELMER [8] package is used for these analysis. The next step is clustering these genes based on the biological role that we investigate which is immune escape for this study. We first investigate literature to gather genes involved in immune-escape. We used these genes to define a basis and then measured semantic similarity relative to this basis. The semantic similarity is defined using GO term similarity (all "MF","BP" and "CC" aspects included). Similarity calculation is based on feature vectors. These vectors are calculated based on semantic distance to each prototype gene. Use of prototype genes creates a non-orthogonal basis for protein vectors. Detailed information on this process can be found in GoSim package which is used in this step [9].

After semantic similarities calculated between all candidate genes we apply the hierarchical clustering. With this clustering we aim to create the clusters that includes genes similar to specific immune-escape functions. The quality of clusters controlled using the silhouettes.

The final step in the computational analysis is enrichment and pathway analysis. There are three different approach exits in the literature. The first one is the over-representation analysis which can be defined as a hyper-geometric test which reveals enriched pathways or ontological entities. As other methods established on statistical tests, a p-value threshold is needed to use for this method which means a pathway that is slightly over the threshold will annotated as insignificant even if it has a high biological significance. Moreover, the method evaluates each gene and pathway without their interactions which might be exists in the biological context. The second approach is the functional class scoring that evaluates functionally related genes to reveal related abnormalities even though they each have little changes in their expression values. This method also does not consider interactions between genes and pathways. The third approach is pathway analysis methods which does not have these shortcomings [10]. We choose pathfindeR which is a pathway analysis method [11]. The method searches for active subnetworks which are well-defined phenotype-associated sets of interacting genes. The steps of the method can be summarized as; (1) Relating the differential genes to subnetworks. (2) Filtering the subnetworks. (3) Doing the enrichment on filtered subnetworks. This process is visualized in Figure 1 (taken from [11] with permission).

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