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Survey on Cell Segmentation and NC Ratio Analysis for Third Harmonic Generated Microscopy Virtual Biopsy Images

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ABSTRACT: Biopsy procedure needs invasive tissue removal from a living subject to determine the occurrence or extent of the diseases. It is complicated and time consuming processes. Third Harmonic Generated Microscopy is a new exemplar for non-invasive *in vivo* virtual biopsy which has the ability to obtain tissue images without removing tissues. The aim of this paper is to discuss about various virtual biopsy microscopic modalities and also about automatic cell segmentation approaches such as watershed transform and convergence index filter, which provides high accuracy for non-invasive analysis of cell Nuclear-to-cytoplasm ratio (NC ratio). NC ratio plays a vital role in identifying or detecting early disease symptoms such as skin cancers during medical imaging analysis.

Keywords: Biopsy, Third Harmonic Generated Microscopy, Cell segmentation, Watershed transform, Convergence index filter, Nuclear-to-Cytoplasm (NC) ratio.

I. INTRODUCTION

Detection of early skin cancer is done by a medical procedure named biopsy [2], which involves the removal of tissues from a living object. The removed tissue is processed by an extensive preparation procedure which includes fixing, embedding, sectioning, staining and placed under a microscope for pathologist examination. Errors may occur during tissue processing and it leads to in accurate diagnosis. It is painful, side effects may also occur like infection and spreading of cancer cells. Optical virtual biopsy techniques for cells and tissues imaging provides capable microscopic details about the benign and malignant lesions without tissue removal. This non-invasive *in vivo* virtual biopsy avoids or minimizes the above mentioned disadvantages involved in virtual biopsy procedure. It also reduces the cost and time consumption in traditional biopsy procedures.

Various non-invasive imaging techniques such as con-focal microscopy [2], two-photon fluorescence (2PF) microscopy, and second harmonic generation (SHG) microscopy have been developed and applied for in vivo human skin diagnosis. Skin disease changes may occur in the deep dermis layer of the skin i.e., several hundred microns below the skin surface. Above mentioned techniques are limited by photo damage, lower resolution, lower penetrability or low contrast. Higher harmonic generation microscopy (HHG) [1], which combines the second and third harmonic generation modalities based on 1230-1250 nm, and can provide high penetration, high resolution and rich contrast. Second harmonic generation (SHG) light nearly disappeared beyond the depth of 200 µm, and the image produced by the SHG also becomes out of focus and lose sharpness at a depth of 250 µm, but still visible at a range of 350 µm.

THG gives a clear boundary definition between the cell nuclei and cytoplasm [1]. Nuclear-to-Cytoplasm [1] ratio plays a vital role in identifying early symptoms of diseases like skin cancer, whose ratio is generally larger in skin cancer than in normal cells and also, provides information about the type and stage of the disease. Several cell segmentation algorithms have been established for describing the exact position of the round objects and gives information about the size, shape and area to obtain useful properties. Image thresholding [4]-[6] is one of the cell segmentation methods used to segment the objects out of background; it lacks adaptability for global thresholding. This method is computationally expensive and does not consider clustered cells i.e., it cannot separate the touching nuclei. Watershed based- segmentation [4], is a popular morphological image segmentation tool, and often produces over-segmentation due to false markers. To reduce over-segmentation fragment merging [6] and marker-controlled [7]

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watershed transform are used. Fragment merging [6], combines the compactness score and probability density function (PDF) score to obtain more correctly segmented nuclei but, it is sensitive to the size of nuclei. Marker-controlled watershed [7]-[8], replaces the region minimum with predefined markers, each of which represents the object but, the difficulty in performing this method is marker extraction. Convergence index filter [9]-[10], degree of convergence is based on the distribution of the gradient vector not on their magnitude. It is based on the maximization of the convergence index at each point of the spatial co-ordinates. Some of the convergence index filters are COIN filter, Adaptive Ring filter, IRIS filter, Sliding Band filter (SBF). Support region, is the major difference among these filters. In this paper [1], we discuss about how marker-controlled watershed transform combines with sliding band convergence index filter works for the automatic cell segmentation in vivo virtual biopsy images of human skin.

II. IMAGE ACQUISITION BACKGROUNDS

Almost all the parts of our body is completely covered by skin, to precisely diagnose the skin diseases, biopsy is the most common method used today. But, it is invasive, painful to the patient and may also risk patient's life by causing infection or spreading of cancer cells. And, it consumes time for fixing, embedding and staining or pathological analysis. For early diagnosis of skin diseases invasive physical biopsy procedures are removed by non-invasive in vivo virtual biopsy. Confocal microscopy [2], Two photon fluorescence (2PF) microscopy, Second Harmonic Generation (SHG) microscopy, Higher Harmonic Generation (HHG) microscopy, which combines the second and third harmonic generation (THG) microscopy are some of the non-invasive in vivo virtual biopsy imaging techniques.

A. CONFOCAL AND TWO PHOTON FLUORESCENCE MICROSCOPY

For obtaining high resolution biological tissue images confocal microscopy method is used [11]. It has the capability of obtaining 3-D image reconstruction from successively obtain 2-D images. It requires a high-numerical-aperture (NA) objective lens to obtain high resolution optical sectioning images. So, the conventional confocal microscopes are huge in size and mostly used for cell culture. Hyejun Ra et al. (2007) proposed a 2-D micro electro-mechanical systems (MEMS) scanner that enables dual–axes confocal microscopy well suited for miniaturization and integration into endoscopes for in vivo imaging [11]. Due to its difficulty in bringing the interesting tissues in contact with the microscopic objective confocal imaging [12] in epithelial tissues other than skin is limited. Kung-Bin Sung et al. (2002) proposed a fiber-optic confocal reflectance microscope for real time human skin imaging used in inner organs of the body. Major disadvantage of confocal microscopy is photo-bleaching, which complicates image acquisition and analysis. In Two-Photon-Excited Fluorescence [13], the interacted matter absorbs two photons and then emits a single-fluorescence photon of higher energy and provides larger penetration depth due to the use of longer wavelength excitations. TPEF can be constrained by inefficient or non- specific dye labelling. Terry B. Huff et al. (2008) proposed a powerful tool, multimodal nonlinear optical (NLO) imaging, which integrates different imaging modalities such as two-photon-excited fluorescence, sum frequency generation and coherent anti-stokes Ramen scattering for imaging different biological structures [13].

B. SECOND HARMONIC GENERATION MICROSCOPY

In in-vivo virtual biopsy second-harmonic generation (SHG) microscopy plays a vital role, used for the study of dermal collagen fiber in skin [3]. SHG [3] is a nonlinear process, related to the interaction of intense light with matter i.e., it is the process of generating light wave, which is twice the frequency of the original wavelength and the generated SHG intensity depends on the square of the incident light intensity. SHG [14] is a high-resolution nonlinear optical imaging microscopy for cellular membranes and intact tissues and it uses a different contrast mechanism additional to TPEF. SHG [3] signals almost disappeared beyond a depth of 200μm and the images become out of focus and lose sharpness and the signals still detectable even at a range of 350μm. Higher Harmonic Generation (HHG) microscopy [1], which combines the second and third harmonic Generation microscopy and it is used for clinical dermatology studies, skin disease diagnosis, and screening of skin conditions.

C. THIRD HARMONIC GENERATION MICROSCOPY

Gwo Giun et al. (2013) proposed an automatic cell segmentation [1] approach for analysing nuclear-to-cytoplasm (NC) ratio for third-harmonic generated virtual biopsy images. Third-Harmonic Generation (THG) [1] is a nonlinear process, related to the interaction of light with matter, which generates light waves with three times the frequency of

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the source. It obeys the laws of conservation of energy and involves virtual electron transitions alone. Hence, there is a possibility of less photo damage and photo bleaching and no energy deposition on the interacted matters. THG [3] signals measured on the chicken skin, muscle and fat and stronger THG signals have been found in the skin. Based on that, THG microscopy applied in wide areas, such as the microscopic imaging of plant leaf cells, cultured cells, live zebra-fish embryos and lives mouse skin. THG arises from cell membrane, cytoplasm organelles, haemoglobin, elastic fiber and lipid bodies and it is mainly subscribe by the cytoplasm of the keratinocytes, boundaries of collagen fibers and red blood cells where SHG provides the collagen fibers in the dermis.

III. CELL SEGMENTATION TECHNIQUES

Nuclear-to-Cytoplasm [1] ratio analysis is a method to identify early symptoms of diseases like skin cancer, whose ratio is generally large in skin cancer cells than in normal cells. Therefore, there are several cell segmentation techniques, which help us to measure the NC ratio. Some of the techniques have been discussed below.

A. IMAGE THRESHOLDING

Image thresholding [4]-[6] is one of the cell segmentation methods used to segment the objects out of background and it does not separate the touching nuclei. In cell images global threshold minimizes the variations between different images which are important for separating the touching or overlapping nuclei but local threshold provides the most suitable value for segmentation with better adaptability. Bin Fang et al. (2003) proposed a two-stage tumor cells identification strategy [4]-[6], in its first stage it uses local adaptive threshold for automatic potential tumor cell segmentation. Here, the global threshold [4]-[6] is very expensive and the clumped cells cannot be considered, because it uses single threshold level for entire image. In global threshold, 'brighter' backgrounds are misclassified as cells and 'darker' cell regions are misclassified as background. By minimizing the negative effect of background noise, local adaptive threshold segments the region of interest from background. Local threshold allows different values, which have been applied to each pixel.

B. WATERSHED SEGMENTATION

In image segmentation, watershed segmentation is the most popular tool and it uses region minimum as starting points. For nuclei segmentation, watershed algorithm is the most widely used one. Watershed algorithm [4]-[8] usually leads to over-segmentation, because it is difficult to have one to one correspondence between regional minima and nuclei. When nuclei are clustered, it becomes worse. Watershed algorithm is used to segment the touching objects, sometimes leads to over-segmentation. To reduce over-segmentation fragment merging [6] and marker-controlled [7] watershed transform are used. Fragment merging [6], which combines the score of compactness and probability density function (PDF). The limitation of probabilities of fragments is that it is mainly based on the training dataset except that it is difficult. Chanho Jung et al. (2010), considers the clustered nuclei segmentation as its major problem and it tries to solve it based on some prior knowledge such as shape of nuclei.

Marker-controlled watershed segmentation [7]-[8], formulates the segmentation as a marker extraction problem, here the nuclei initially considered as markers. To obtain accurate markers mathematical morphology condition erosion is used, which effectively segments the clustered cells with less over-segmentation. In this segmentation method, the regional minimum is replaced by the predefined markers which represent the object. Marker [8] is a connected component of an image, which represents existence of an object. Instead of flooding from the regional minima, marker-controlled watershed floods from the markers. It is important to extract the correct markers because it may leads to over-segmentation or under-segmentation. Xiaodong Yang et al. (2006), proposed a new marker extraction method based on the condition erosion. Fine erosion and coarse erosion structures [8] are the two masks designed based on the cell shape. Coarse erosion structure [8] tends to remain the actual shape when reducing the size of clustered cells. Fine erosion structure [8] leads to the loss of shape information, results in under-segmentation.

Gwo Giun (Chris) Lee et al. (2013), proposed [1] a automatic cell segmentation approach based on watershed transform with marker controlled strategy, which cannot be directly applied on the gradient map due to over-segmentation. Here, nuclei initialization [1] would prevent the over-segmentation by applying the marker controlled strategy after minima imposition. Internal markers are used to determine the potential nuclei and the external markers are used to obtain respective cell boundaries. To obtain the internal marker blob detection followed by outlier removal

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carried on and for external maker distance transform takes place, each internal marker must contain only one external marker. Followed by nuclei segmentation, cytoplasm segmentation takes place.

C. CONVERGENCE INDEX FILTER

For cytoplasm segmentation convergence index filter [9] is used. Convergence index [9]-[10] is a measure of how strongly the gradient vector point towards the pixel of interest. Convergence index filter [9]-[10] degree of convergence is based on the distribution of the gradient vector not on their magnitude. This filter evaluates the degree of convergence of the gradient vectors in the neighbourhood of each pixel of interest, i.e., the distribution of the directions of the gradient vectors with respect to the pixel of interest is evaluated [9]. Hidefumi Kobatake et al. (1999) proposed a unique filter called an iris filter, which evaluates the degree of convergence of the gradient vectors within its region of support toward a pixel of interest [9]. This filter changes its shape and size based on the gradient vectors distribution in the pixel of interest. Regardless of the contrast of its background, it is possible to enhance indistinct boundaries and detect rounded convex regions in an image [9]. It is based on the maximization of the convergence index at each point of the spatial co-ordinates.

Some of the convergence index filters are Coin filter (CF), Adaptive Ring filter (ARF), IRIS filter (IR), Sliding Band filter (SBF). Support region, is the major difference among these filters [10]. The CF uses a circle with variable radius as support region, the IF maximizes the convergence index by adapting the circle's radius value on each direction and the ARF uses a ring shaped region with fixed width and varying radius [10]. Finally, the SBF combines the ideas of IF and ARF by defining a support region formed by a band of fixed width , whose position is changed in each direction to allow the maximization of the convergence index at each point [10]. The SBF filter is normally well suited for cell detection because of its overall convex shape. Here, we assume that both cell's nuclei and cytoplasm gradient convergence center is same. This makes use of nuclei's information to guide cytoplasm segmentation.

IV. NUCLEUS TO CYTOPLASM RATIO

The Nucleus-to-Cytoplasm ratio is the ratio of the nucleus size to the cytoplasm size. During cell division, the nucleus makes up the larger portion of the cell. The ratio gets decreases when the cell start matures and the cancerous cell does not get mature and healthy. So, the Nucleus-to-Cytoplasm ratio is always larger in mature normal cells. This NC ratio can be used to diagnose the cancer cells in some tissues, by using this ratio we can also determine the skin age and it is used for many medical applications.

V. CONCLUSION

Traditionally, early detection of skin cancer is diagnosed by physical biopsy procedures, which involves invasive tissue removal causes pain and also time consuming. In this context several non-invasive in vivo virtual biopsy techniques have been discussed. In this survey, we have also discussed some of the cell segmentation techniques such as image thresholding, watershed segmentation, which involves fragment merging and marker-controlled watershed segmentation and convergence index filters. Based on cell's nuclei and cytoplasm segmentation analysis of Nucleus-to-Cytoplasm (NC) ratio, which plays a vital role in early detection of skin cancer also have been discussed.

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