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Targeting Cell Nuclei for the Automation of Raman Spectroscopy in Cytology

Jonathan Blackledge, Dmitri Dubovitskiy and Fiona Lyng

Abstract—Biological cell analysis has, and is still, an important aspect in medical research and clinical diagnosis. Although cytologists routinely undertake a diagnosis using optical microscopy, human factors make this routine unreliable especially when it involves many consecutive tasks that are monotonous, time consuming and focus on pattern matching tasks where the patterns concerned are not always entirely clear and/or do not necessarily belong to a well defined class. Raman Spectroscopy provides the potential to generate a fundamental representation on the status of cellular conditions through the characteristics of a Raman spectrum generated by the back-scatter of a laser pulse incident on the cell nucleus. However, this approach requires the nucleus of the cell to be accurately targeted from a complex of many hundreds of such cells within a conventional optical field of view as defined by the resolving properties of a microscope. This requires specialist digital image processing methods to be developed and in this paper we discuss a new approach to the processes of object detection, recognition and classification for target detection in cytology using Raman Spectroscopy. In particular, we report on a system designed for the inspection of slides used in a cervical cancer screening system known generally as a ‘Pap-smear’ test. After providing a short introduction to the pattern recognition in general, we present a unique procedure for automating the targeting process based on an analysis of the principal issues associated with object recognition which include the basic model used and segmentation algorithms derived from the model.

Index Terms—Pattern analysis, segmentation, object recognition, image morphology, fuzzy logic, cervical cancer screening, Raman spectroscopy

I. INTRODUCTION

Approximately 471,000 women are diagnosed with invasive carcinoma of the cervix each year and 233,000 die from the disease, worldwide. Cervical cancer is among the most common female cancers in many countries of the developing world. Sexually transmitted infection by certain strains of the human papilloma virus is a major cause of cervical cancer; smoking has also been linked to the disease. In Ireland, for example, on average 800 women are diagnosed with cervical pre-cancers and 180 women are diagnosed with cervical cancer. It is estimated that 2,900 women are living with cervical cancer and 31,200 women have had cervical pre-cancer. The incidence of Gynaecological cancers has been predicted to increase by 98% by 2020 (from 855 to 1676) [37] A National Cervical Screening Programme, Cervical Check, was launched in September 2008 and initially the Irish government outsourced the screening to Quest Diagnostics, US. In April 2010, Sonic Healthcare, the largest pathology laboratory provider in Australia/New Zealand and Europe and the third largest provider in the United States were awarded half of the screening contract and have since set up a pathology laboratory in Dublin. The technology described in this paper, if shown to be accurate in future clinical trials, could provide a cost effective and high-throughput screening system which could influence the future of screening services world-wide.

Cervical cancer is preceded by a precancerous condition called CIN (Cervical Intraepithelial Neoplasia) which can be easily treated if detected. It is therefore important to identify CINs by screening women. The screening test is called a cervical smear. A clinician removes a small sample of cells from the surface of the cervix and spreads the sample onto a glass slide, the material being ‘fixed’ in alcohol. The slide sample is typically stained and examined using an optical microscope, reports being provided on any abnormal cells or cell clusters. This technique has a false negative error rate of 15-59% [4]. There is undisputed evidence that cervical cancer is related to human papillomavirus(HPV) infection [5]. Despite the recent introduction of Gardasil and the planned introduction of Cervarix, both HPV vaccines, routine cervical screening is still recommended as the vaccines do not protect against all high risk HPV strains and some women may not benefit from the vaccines if there is a pre-existing infection or if they do not receive the complete number of doses.

Although staining the sample can provide valuable colour identifiers for automatic image recognition, the approach discussed in this paper is entirely stain independent due to the influence that staining has on the generation of a Raman spectrum [6]. Colour independent pattern recognition methods are therefore considered to detect and target abnormal cells (in particular the cell nucleus). The use of automated recognition systems of the type reported here has the potential to provide a new level of stability and robustness in screening procedures, thereby making national cervical screening programmes a possibility in countries where they are not currently practically viable.

II. CURRENT METHODS

Currently, a Papanicolaou (Pap) smear is used to screen for Cervical Intraepithelial Neoplasia (CIN) and cervical cancer in the general female population. There are two main approaches; manual screening and automated screening. The Papanicolaou
test (also called Pap smear, Pap test, cervical smear, or smear test) is a screening test used to detect cancer and pre-cancer in the ectocervix. A tool is used to collect the cells from the cervix and after placing on a slide and staining the cells are examined for abnormalities under the microscope by highly trained personnel. The Pap test is an effective, widely used method for early detection of cervical cancer and pre-cancer. However, it is widely acknowledged that sensitivity values are low (with sensitivity and specificity values of 72% and 94% , respectively [7]). Since the mid 1990s, liquid based cytology has been introduced where the cells are placed in a liquid medium which preserves the cells and this sample is processed in the cytology laboratory into a monolayer of cells on a slide. The sample is then stained and examined by microscopy and by highly trained personnel as before. There are two main types of liquid based cytology, SurePath (TriPath Imaging / BD Biosciences) and Thin Prep (Cytex Corp / Hologic).

A more recent innovation is the introduction of automated imaging systems (ThinPrep Imaging System or Focal Point Slide Profiler) that automatically scan the slide for large and dark nuclei which are features of abnormal cells. Generally the slide is scanned twice, once by the imaging system and then manually by the cytologist. The imaging system identifies areas of interest based on cellular DNA content that are subsequently reviewed by the cytologist. The ThinPrep Imaging system has been shown be at least equivalent to manual screening [8] while some diagnostic areas demonstrated an increased sensitivity and specificity [9]. A marked improvement in productivity has consistently been found. After automated screening each sample is reviewed by a cytologist and if any abnormalities exist, the sample is passed to the Chief Medical Scientist and then to a pathologist. This means that each sample is screened at least twice and potentially four times if there is a suspected abnormality. Despite the introduction of automated imaging systems, some clinical laboratories even choose to manually screen each sample in addition to the automated screening.

Raman spectroscopy is a powerful tool that can generate a biochemical fingerprint of a sample in a rapid and non-destructive manner. It is sensitive to subtle biochemical changes occurring at the molecular level allowing spectral variations corresponding to disease onset to be detected. There is convincing evidence that Raman spectroscopy can be used as a diagnostic tool to identify spectral changes in malignant and premalignant cells. In recent years, Raman spectroscopy has been used in the detection of a variety of cancers including, breast, lung, brain, colon, liver, oral, oesophageal, prostate and cervical cancer [1], [2]. However, finding the abnormal cells prior to Raman spectroscopy can be challenging and can require specialist personnel to correctly identify the abnormal cells. In order to automate the approach, a system which combines Raman spectroscopy with novel automated image processing is required. This cell detection approach can provide more complete information about whole cells. Further, a learning algorithm based on a pathologists/cytologists experience and expertise can also be employed to provide a greater degree of accuracy for the system. The specific advantages of such a technology are:

- higher throughput and increased speed due to automated cell detection;
- higher accuracy due to Raman analysis of biochemical fingerprints;
- significantly reduced work-load resulting in lower costs to health service providers.

In order to accomplish this, advanced pattern recognition for optical microscopic images are required based on a suitable imaging model.

### III. PATTERN RECOGNITION

Pattern recognition is a component of image analysis which involves the use of digital image processing methods designed in an attempt to provide a machine interpretation of an image, ideally, in a form that allows some decision criterion to be applied [12], [13]. It uses a range of different approaches that are not necessarily based on any one particular theme or unifying conceptual framework. This is because there is no complete and unique theoretical model available for explaining and simulating the processes of human visual image comprehension. Hence, machine vision remains a subject area in which automatic inspection systems are advanced without having a fully operational theoretical framework as a guide. For this reason, numerous and non-related algorithms for understanding two- and three-dimensional objects in a digital image have and continue to be researched in order to design systems that can provide reliable automatic object detection, recognition and classification in an independent environment (e.g. [14], [15], [16] and [17]).

Pattern recognition can be thought of as the process of linking parts of the visual object’s field with stored information or ‘templates’ with regard to a pre-determined significance for the observer. There are a number of questions that need to be considered in the development of any machine vision system. These include: (i) what are the goals and constraints? (ii) what type of algorithm or set of algorithms is required to complete the system? (iii) what are the implications for the processes, given the types of hardware that might be available? (iv) what are the levels of representation required? The levels of representation are dependent on what type of segmentation process can and/or should be applied to an image. These are recorded as place tokens and stored in a database. Regions of pixels with similar intensity values or sets of lines are obtained by isolating the edges of an image scene and computed by locating regions where there is a significant difference in the intensity. Such sets are subject to inherent ambiguities when computed from a given input image and associated with those from which an existing data base has been constructed. These ambiguities can only be overcome by the application of high-level rules based on how humans interpret images, but the nature of this interpretation is not always able to be clearly defined. Parts of an image may tend to have an association if they share size, figural similarity, continuity, shading and texture. For this reason, it is necessary to consider how best to segment an image and what form this segmentation should take. For example, optical microscopy involves the use of image processing methods that are often designed in an
attempt to provide a machine interpretation of a biological image, ideally in a form that allows some decision criterion to be applied, such that a pattern of biological significance can be recognised [12], [13]. Compared to image processing, computer vision is more than automated image processing. It results in a conclusion, based on a machine performing an inspection of its own. The machine must be programmed to be sensitive to the same aspects of the visual field as humans find meaningful. In this context, segmentation is concerned with the process of dividing an image into meaningful regions or segments. It is used in image analysis to separate features or regions of a pre-determined type from the background and it, in most cases, the first step in automatic image analysis and pattern recognition. Segmentation is broadly based on one of two properties in an image: (i) similarity; (ii) discontinuity. The first property is used to segment an image into regions which have grey (or colour) levels within a predetermined range. The second property segments the image into regions of discontinuity where there is a more or less abrupt change in the values of the grey (or colour) levels.

Pattern recognition can be considered to be a form of machine understanding based on assigning a particular class to an object. The tasks of construction and application of formal operations for numerical or character representation of objects in a real or idealized world is the basis for pattern recognition. This depends on establishing equivalence relations that express a fit of evaluated objects to any class with independent semantic units. The recognition classes of equivalence can be set by the user in the construction of an algorithm, which uses selective representations or external padding information on a likeness and difference of objects in the context of a solved task. This is the basis for phrase ‘recognition with the teacher’. For a typical object recognition system, the determination of the class is only one of the aspects of the overall task. In general, pattern recognition systems receive data in the form of ‘raw’ measurements which collectively form a stimuli for the generation of a ‘feature’ vector [18], [19]. Uncovering relevant attributes in the elements present within the feature vector is an essential part of such systems. An ordered collection of relevant attributes which most clearly represent the underlying features of the object is assembled into the feature vector. In this context, learning amounts to the determination of the rules of associations between the features and attributes of a pattern.

A. Practical Image Recognition Systems

Practical image recognition systems generally contain several stages in addition to the recognition engine itself. The recognition represents information processing that is realised by some converter of the information having an input and output. On input, such a system establishes information on the properties of an object. On output, the information shows which class or feature of an object is to be assigned.

When a computerised system decides on the task of classification without engaging external learning information, it is called automatic classification - ‘recognition without the teacher’. The majority of algorithms for pattern recognition require the engagement of a number of computational procedures which can be provided only with high-performance computer equipment [20].

There are two principal methods for object recognition using either a parametric or non-parametric approach. Statistical voting and alphabetic proposition methods have been reviewed in [21], [22] and [23]. The main disadvantage with this method is that classes have to be clearly defined so that no overlapping is allowed. Methods based on a principal of separation and potential functions can be found in [36] and [16]. These methods require a large amount of training data or preliminary information about system to be generated which makes the recognition process less flexible. In general, there is no method or, at least, an operation system, which considers objects from the point of view of a superposition of global scenery. This leads to the following problem: how can we evaluate an object in terms of it being part of the ‘bigger picture’ without losing specific details on its particular texture for precise recognition? This includes the incorporation of concepts from Fractal Geometry [38], [39], [40], and [41] and Fuzzy Logic [44], [45] and [46].

B. About this Paper

The main technical objectives associated with the approach developed in this paper are:

- To develop image processing algorithms to identify suspicious cells in a cervical smear sample;
- to develop an Application Program Interface (API) to control a Raman microscope and integrate this with Raman spectrometer and image processing software;
- to develop a user friendly Graphical User Interface;
- to test the system for automated cervical cancer screening.

In this context, we consider an approach to object detection in an image scene that is based on a new segmentation algorithm using a Contour Tracing Algorithm [48] and a Space Oriented Filter. Because some parts of the image need enhancement, a novel self-adjustable filter for isolated feature sharpening is also developed. The segmented object is then analysed in terms metrics derived from both a conventional Euclidean geometric and textural perspective, the output fields being used to train a fuzzy inference engine and the recognition structures being based on some of the technologies for image processing, analysis and machine vision reported in [23], for example. The approach considered is generic in that it can, in principle, be applied to any type of imaging modality used to develop a membership function. The unique approach reported in this paper (which represents the main contribution to the field) is to develop a focal membership function, obtained by focusing a microscope at different levels in the image plane. It is this approach that is used to differentiate the nuclei of cells from the surrounding material in the image plane and thereby generate the target required. However, to place this approach and the methods developed in the context of the application required, we begin with a short overview of Raman Spectroscopy in Cytology.
IV. RAMAN SPECTROSCOPY IN CYTOLOGY

A Raman spectrogram is generated by the inelastic scattering monochromatic (laser) light and is characteristic of the vibrational, rotational, and other low-frequency modes of a material that has been penetrated by the light (it is a volume scattering effect). The interaction of the molecular structure with laser light, yields photons with different energies. This results in the generation of a wavelength spectrum that is characteristics of the vibrational modes of the molecular structure of a material. The ‘material’ can include biological entities such as cells and cell nuclei. Raman scattering is a form of weak scattering and a principal task in Raman spectroscopy is to separate the elastic scattered light from the inelastic ‘Rayleigh scattered’ light which is a dominating effect [25]. Compared to Rayleigh scattering (i.e. an inelastic volume scattering process in which the material properties are taken to be inhomogeneous), modelling inelastic scattering processes such as Raman scattering is significantly complex. Nevertheless, even in the absence of a full working physical model, if the characteristics of a Raman spectrum can be correlated with material states that are biologically significant, and, this correlation is statistically significant from one measurement to the next, then the method can be used effectively for diagnostic purposes.

Raman spectroscopy has been used in the detection of a variety of cancers [1], [2] including cervical cancer, [24], [26] and [27], and shown to be a powerful diagnostic tool for cervical tissue sections and cervical smear samples, e.g. [28], [29] and [30]. This clinical application of Raman spectroscopy for cervical cancer has been the subject of both a UK and International Patent [31]

Figure 1 shows a typical example of the type of Raman spectra that are generated by cell types in a cervical cytology sample. Subtle differences in the signature associated with Raman scattering from nucleic acids, proteins and lipids can be observed. It is these signatures that form the basis of Raman spectroscopy in cytology.

Irrespective of the spectral analysis algorithm(s) used, a principal problem is to maintain consistency so that the results are statistically significant, a significance that predicated on a physically self-consistent system. This includes targeting the cell nuclei automatically. In this respect, and, in terms of a clinical application, the ideal instrument for this purpose is a microscope based system where the user ‘points and clicks’ on an area of interest, whereby a spectrum or a number of spectra from individual cells/tissue of the type illustrated in Figure 1 are automatically recorded. These spectra can then be compared to a large library of pre-recorded spectra from a wide sample base including all grades of cervical intraepithelial neoplasia (CIN I, II and III). An algorithm is then used to classify the spectra into the most appropriate group and a clinically significant classification returned via a simple user interface. This includes the introduction of a learning algorithm based on the experience and expertise of a pathologist/ cytologist which can be employed to provide a greater degree of accuracy, abnormal cells being identified using image processing algorithms which are subsequently targeted for Raman spectral analysis.

There are a variety of ways in which the spectral signatures illustrated in Figure 1 can be classified providing consistency is maintained from sample to sample. For example, Figure 2 shows a Principal Component Analysis [33] for Raman spectra that, in this example, provides point clusters associated with the coordinates of the first three Principal Components. This analysis provides the basis for implementing the approach adopted in [34], for example, which applies ‘Fuzzy Logic’ to categorise parameters that include those associated with signals derived from the boundaries of ‘fuzzy objects’ in medical images.

The rationale for the research reported in this paper is to develop a second generation system which eliminates the need for the pathologist/ cytologist to ‘point and click’. The system uses image processing algorithms to identify ‘suspicious cells’ in a cervical smear sample. One approach is to exploit the well known fact that the cell nuclei increases in size in abnormal cells because of increased DNA content. However, the same effect can be used to identify suspicious cells by detecting epithelial cells in the mixed cell population of a cervical smear. As illustrated in Figure 3 [35], a number of different cell types, including red and white blood cells, can be present as well as the squamous epithelial cells. The DNA content increases in abnormal cells resulting in larger and denser nuclei and
an increased nucleus-to-cytoplasm ratio. Using this effect, the epithelial cells can, in principle, be detected on the basis of their larger size and an image created showing all the detected epithelial cells which is then saved with a patient identifier code. In this respect, if the nucleus-to-cytoplasm ratio and optical density of nuclei are measured in cervical smear samples then the spatial co-ordinates of the suspicious cells can be recorded. Using these co-ordinates, Raman spectra can be obtained from each suspicious cell and compared to the library and classified.

Although a feasible solution for stained samples (where there is usually clear colour contrast between the cell nuclei and the cytoplasm), for application to Raman spectroscopy, this approach cannot be taken due to the effects that staining has on the Raman spectrum. Even if the approach can be adapted from non-stained samples, the processing time required to obtain the coordinates of a cell with a nucleus to cytoplasm ratio that is ‘larger than normal’ can be excessive, requiring specialist image processing hardware.

\[ \text{ThinPrep slide feature identified by the three blue rings shown in Figure 4.} \]

Image acquisition depends on the technology that is best suited for integration with the particular application. For pattern recognition in histopathology, for example, high fidelity digital images are required for image analysis whose resolution is, at least, compatible with the image acquisition equipment used for human inspection, e.g. an optical microscope. The microscope used for analysing a cervical smear slide has to be equipped with a ‘C-Mount adapter’ and digital camera. The images used in the current application as discussed in this paper are, in general, relatively noise free and are digitised using a standard CCD camera. Nevertheless, it is important that high fidelity images are obtained which are homogeneous with regard to brightness and contrast through application of an optical diffuser, for example. Unless consistently high quality images can be generated that are compatible with the sample images used to design a given computer vision system, then that same system can be severely compromised.

The cells obtained via a cervical smear do not cover the whole surface area within the circle given in Figure 4. A low magnification lens is therefore used to segment regions of interest which include cell clusters where there is a high population density of cells that can be be inspected. The focus is on the identification of the edge features which is an important component of cell recognition, in general. This identification provides information on the basic topology of a feature from which an interpretative match can be achieved. Some edges can be detected only in terms of a representative view of a whole image and have no connection with local pixels. Nevertheless, the segmentation of an image into a complex of edges is a useful pre-requisite for object identification and the solution requires an analysis of the whole scene.

Although many low-level processing methods can be applied for this purpose, the problem is to decide which object boundary each pixel in an image falls within and which high-level constraints are necessary. In many cases, a principal question is which comes first, recognition or segmentation?

\[ \text{V. TECHNOLOGY OVERVIEW} \]

In this section, we briefly review the principal components associated with the application. Cytological cells are prepared using the ThinPrep technology and ‘fixed’ on a slide of the type shown in Figure 4. The cell sample is fixed as a monolayer within the red circle shown in Figure 4. Each slide has a unique Identification Number and a OCR (Optical Character Recognition) system controls the ‘order’ of the slides. The slides are stored in a cartridge and loaded into an optical microscope. For the work reported in this paper an Olympus BX51 microscope is used together with a Prior motorised stage which selects and scans the slides. The principal purpose of the image recognition system developed to date is to return the relative coordinates that define a target (suspect) cell, in particular, the nucleus of that cell. For this purpose each slide needs to be calibrated. This begins by searching for three features allocated to each slide which form part of the standard

\[ \text{VI. IMAGING MODEL} \]

Suppose we have an image which is given by a function \( f(x, y) \) and contains some object described by a set (a feature vector that may be composed of integer, floating point and strings) \( S = \{s_1, s_2, ..., s_n\} \). We consider the case when it is necessary to define a sample which is somewhat ‘close’ to this object. This task can be reduced to the construction of some function determining the degree of proximity of the object to a sample - a template of the object. Recognition is the process of comparing individual features against some pre-established template subject to a set of conditions and tolerances. The process of recognition commonly takes place in four definable stages: (i) image acquisition and filtering (as required for the removal of noise, for example); (ii) object location (which may include edge detection); (iii) measurement of object parameters; (iv) object class estimation.

For the current application, cell location is undertaken via the computation of a set of weight coefficients \( k_{x,y} \) that, for
each pixel are defined in terms of the equation
\[ f_{m,n} = f(x,y)k_{x,y} \]
where
\[ k_{x,y} = \left[ \frac{1}{f(x,y)} \begin{bmatrix} k_{x-1,y+1} & k_{x,y+1} & k_{x+1,y+1} \\ k_{x-1,y} & p_{x,y} & k_{x+1,y} \\ k_{x-1,y-1} & k_{x,y-1} & k_{x+1,y-1} \end{bmatrix} \right] \otimes p_{\text{obj}}(x,y) \]
and \( \otimes \) denotes the convolution integral (over both \( x \) and \( y \)), the matrix values being user defined. This result yields local dependency between the current pixel \( f_{m,n} \) and the object pixels, global evaluation being determined by \( p_{\text{obj}}(x,y) \) which is the probability that the pixel could be a part of an object. This probability is calculated from a Fuzzy Logic Membership Function which has a feed-back to the current object location. The function \( p_{\text{obj}}(x,y) \) is a two dimensional matrix and recalculates local values dynamically using the object table location \( f_{m,n} \). The construction of this matrix is based on the following procedures:

1. The intensity level of the object(s) is computed. This level uses only those pixels which have not been recognised as a part of the object. The object level, denoted by \( L_{\text{obj}} \), is initially set to be lower than the background level \( L_{\text{bgr}} \), and, as the recognition process continues, so long as \( L_{\text{obj}} \neq L_{\text{bgr}} \), all objects are recognised as having been indexed according to the equation [47] and [48]:
\[ L_{\text{bgr}} = \text{mean}[f(x,y) - f(m,n)] \]
2. In order to obtain \( L_{\text{obj}} \), a probabilistic min-max equation (which has been experimentally tested for different images) is used given by [47]:
\[ L_{\text{obj}} = \begin{cases} L_x, & L_x \leq L_y; \\ L_y, & \text{otherwise}. \end{cases} \]
where
\[ L_x = \frac{1}{2} \left( \max_y \left[ \min_x f(x,y) \right] - \langle \min_x f(x,y) \rangle_y \right) + \langle \min_x f(x,y) \rangle_y, \]
and
\[ L_y = \frac{1}{2} \left( \max_x \left[ \min_y f(x,y) \right] - \langle \min_y f(x,y) \rangle_x \right) + \langle \min_y f(x,y) \rangle_x. \]
In order to maintain simplicity, we do not include in this equation that component which is responsible for dividing those previously defined objects in \( f_{m,n} \). For more complex images, a filter can be used to restrict a region of interest \textit{a priori} depending on the light conditions and point of 'evaporation'. The approach considered is generic in that it can, in principle, be applied to any type of imaging modality. The system developed for the application of cell location includes features that are based on the textural properties of an image which is an important theme in the field of pattern analysis for biophotonics.

In the current application, it is the cell nuclei that need to be identified and are the principle regions of interest. This is because in the application of Raman spectroscopy the cell nucleus is the principal target area [10] and [11]. During the process of cell nuclei recognition, all cells are indexed from high to low probability of abnormality. Raman spectrograms are then generated for those targets with the highest probability of abnormality thereby confirming or otherwise the condition of the cell [6]. The method used for targeting the cell nuclei is discussed in the following section.

VII. TARGETING CELL NUCLEI

The approach reported in this paper is predicated on Liquid Based Cytology (LBC). A clinician takes a sample in the same way as in a PAP test, but using a very small brush instead of a spatula. The head of the brush is broken off and immersed in a small vessel of liquid instead of smearing the sample directly onto a slide. This approach is better at preserving the cells and so the results of the test are generally more reliable. At present, about one in twelve PAP smears have to be repeated because the results are inconclusive due to poor readability. With LBC, far fewer tests have to be repeated. Although re-training programmes are needed to accompany the introduction of this technique, the approach reported in this paper is nevertheless based on the assumption that digital images are acquired from LBC slides.

A. Cell Categories

There are two principal types of cervical cancer: Squamous cell cancer and Adenocarcinoma. They are named after the type of cell that becomes cancerous. Squamous cells are the flat skin-like cells that cover the surface of the cervix. They are the most common type of cervical cancer. Adenocarcinoma cells are glandular cells that produce mucus. The cervix has these glandular cells along the inside of the passageway that runs from the cervix to the womb (the endocervical canal). Adenocarcinoma is the cancer of these cell types. It is less common than squamous cell cancer, but has become more commonly recognised in recent years. Only about one in five to one in ten cases of cervical cancer are adenocarcinoma and are associated with a similar precancerous phase. It is treated in the same way as squamous cell cancer of the cervix.

B. Depth of Focus Analysis

In a simplistic three-dimensional sense, most cells consist of some basic generic features which can be classified. The most important of these is that: (i) the Cytoplasm has a relatively flat textural surface whereas the Nucleus is not flat but has relatively significant depth; (ii) the border between the Nucleus and the Cytoplasm has a distribution of textures in depth, i.e. a border pattern with changes in its textural properties as a function of depth. Images of this ‘depth dependence’ can be acquired by considering different focal depths. An example of this is given in Figures 5, 6 and 7 which show three images...
C. Nuclei Detection using Three-Dimensional Cell Structure

There are two imaging methods that can be considered based on: (i) using a two-dimensional image generated in a single focal plane - the mid-focal-plane, for example; (ii) considering a three-dimensional image obtained by changing the focal plane to yield a set of images at different focal depths. While the first approach is the most conventional it does not necessarily provide the most accurate solution to the current problem as discussed in [42] and [43]. This is because a mid-focal-plane image may not capture the border between the nucleus and the cytoplasm in a way that is unambiguous due to textural three-dimensional characteristics of the nuclei-cytoplasm interface.

The second approach considers the three-dimensional cell structure which, in general, may not be of clinical significance at least in a conventional sense. However, by using this approach to differentiate between the three dimensional nature of the cell nucleus relative to the flatness of the Cytoplasm we show that it is possible to extract the nucleus, detect a region of interest and thereby apply a Fuzzy Logic based Membership Function.

Suppose we have a three-dimensional array composed of a set of images (of the type given in Figures 5, 6 and 7) which is denoted by a function $f(x, y, z)$ and contains some object described by a set of features $S = \{s_1, s_2, ..., s_n\}$. We consider the case when it is necessary to define a sample which is similar to this object in terms of a matching set. A conventional method consists of calculating some function of a point-wise coincidence between the map of the object and the image together with a search for the maximum value of this function. In terms of a ‘similarity function’, this method can be represented in terms of metrics that include the sum of square deviations, the sum of the modulus of deviations or as a pair of sum of multiplications of brightness values (function of the greatest transparency), for example. The first two similarity functions compute the ‘smallness’ of a functional pair (instead of searching for a maximum a search is launched to obtain the minimum). However, in this application, not all fragments of an nucleus edge are equally important and hence, a broadly distributed functional evaluation matched with weighted coefficients is undertaken. The selection of weight coefficients is calculated from a given set of samples with two fuzzy logic sets for the nucleus and for the cell.

Normally, fuzzy logic systems for image analysis provide a decision using a knowledge database by subscribing different edges. In this application the nucleus edge is distributed only in depth and the fuzzy set is defined such that it is not necessary to use a positive feedback learning procedure for second stage object modelling [44].

The computation of a particular value of the Membership Function $p_{obj}(x, y, z)$ is obtained according to the equation

$$p_{obj}(x, y, z) = \int_{xyz} (f_{x,y}L_{obj} - L_{bgr} + edge_{xyz}) dxdydz$$

for a closed border of the object, a schematic diagram being given in Figure 8. The function $edge_{xyz}$ is an edge detection function. In Figure 8, and, by way of an example only, the
maximum value of $p_{\text{obj}(x,y,z)}$ corresponds to the top surface of the nucleus. However, this maximum value may change for different cells because not all cells are fixed in a single layer. Hence, it is necessary to undertake a search for this local maximum. For a three dimensional polygon, the local maximum can occur within the Cytoplasm and thus, the object segmentation function is limited to the locality of a particular region of interest. Irrespective of the area allocation, the algorithm is applied recursively until nuclei fail to be detected. The shapes of these nuclei (as fixed on a slide) are not continuous and so it is not possible to develop a model based on a deterministic logic. However, Fuzzy Logic is well suited for this application.

### D. Membership Function Allocation

We use the approach discussed in the previous section to allocate a Membership Function which provides a fast and reliable solution based on changing the focal depth over a number of consecutive steps $n$ generated by a stepping motor. We consider the function

$$V(L_{\text{bgr}}) = \frac{2\pi (R_{n+1} - R_n)}{Z_{n+1} - Z_n}$$

where $R_n$ is the radius at step $n$ and $R_{n+1}$ is radius at step $n + 1$ computed by taking the average length from the Centre-of-Gravity to the edge of the nucleus segmented using the edge detection function. In correspondence, $Z_n$ is the depth of focus for step $n$ and $Z_{n+1}$ is equivalent at the next consecutive step. By computing

$$dZ = \frac{k}{V(L_{\text{bgr}})}$$

where $k$ is a correction coefficient specific to the stepping motor (obtained by calibration of the microscope) the point when $dZ < \text{Threshold}$ is taken to represent the upper bound of the nucleus. The value of the threshold and the step lengths need to be established experimentally so that they are within the bounds on the extent of the nucleus. The size and shape of the upper surface of the nucleus is mapped on to a reference image for morphological processing.

Two special filters are used for this purpose, namely, the ‘Detour by Object Contour’ and the ‘Convex Hull Spider’ algorithms presented in [48]. These filters are used for generating a uniformly closed boundary to define the edge of the cell nucleus - the ‘edge detection function’. The ‘Centre-of-Gravity’ of the closed boundary is taken to be the centre of nucleus and its coordinates used to target the point at which a Raman spectrum is generated. This is illustrated in Figure 9 which shows a screen shot from the GUI used for targeting a cell nucleus based on the approach discussed in this paper. The computational time associated with the system developed to date depends on the image resolution and normally varies from between three to six seconds for each cell using a MATLAB-Linux environment. For real time recognition, the approach considered can be implemented by means of a FPGA (Field Programmable Gate Array) to yield a computational performance that is compatible to the video frame rate associated with the application.

### VIII. Conclusion

The work reported in this paper is part of a wider investigation into automating the application of Raman Spectroscopy in Cytology with a focus on the detection of abnormal cervical cells using LBC based on [6]. We have been concerned with the task of developing a methodology concerned with two key tasks: (i) the partial analysis of an image in terms of the textural properties that characterise that structure - the cell nucleus; (ii) the use of a fuzzy logic engine to classify the nucleus edge. The specific contribution to pattern recognition considered has been addressed and involves using the texture contrasts that occur in depth between the nucleus of a cell and the Cytoplasm. By exploiting this property it is possible to isolate and thereby target the nucleus of a cell in order to generate a Raman Spectrum. This approach has been developed as part of a scheme to automate the process of Raman spectral diagnosis in Cytology and forms the basis of a British Patent [49] filed in October, 2012.
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