Early Experiences in Mitotic Cells Recognition on HEp-2 Slides

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Abstract

Indirect immunofluorescence (IIF) imaging is the recommended laboratory technique to detect autoantibodies in patient serum, but it suffers from several issues limiting its reliability and reproducibility. IIF slides are observed by specialists at the fluorescence microscope, reporting fluorescence intensity and staining pattern and looking for mitotic cells. Indeed, the presence of such cells is a key factor to assess the correctness of slide preparation process and the reported staining pattern. Therefore, the ability to detect mitotic cells is needed to develop a complete computer-aided-diagnosis system in IIF, which can support the specialists from image acquisition up to image classification.

Although recent research in IIF has been directed to image acquisition, image segmentation, fluorescence intensity classification and staining pattern recognition, no works presented methods suited to classify such cells. Hence, this paper presents an heterogeneous set of features used to describe the peculiarities of mitotic cells and then tests five classifiers, belonging to different classification paradigms. The approach has been evaluated on an annotated dataset of mitotic cells. The measured performances are promising, achieving a classification accuracy of 86.5 %.

1. Introduction

Indirect immunofluorescence (IIF) imaging is the recommended laboratory technique to detect autoantibodies in patient serum, which are at the basis of autoimmune disorders, a chronic inflammatory process involving connective tissues and giving rise to connective tissue diseases (CTDs) [11, 18]. In patients with suspected CTDs, antinuclear autoantibodies (ANAs) dispatched on the HEp-2 cell substrate are used as markers for disease detection [11]. HEp-2 slides are observed by specialists at the fluorescence microscope, reporting fluorescence intensity and staining pattern, and looking also for mitotic cells. Indeed, mitotic cells are added to slides producers in order to guarantee the correctness of the tests and to help the doctors in decision on staining pattern since peculiarities of mitosis cycle match with certain staining patterns rather than others. Hence, such cells permit to be confident with decisions and to assess the reports [16].

In IIF the availability of accurately performed and correctly reported laboratory determinations is crucial for the clinicians, demanding for highly specialized personnel that is not always available. Moreover, the readings in IIF are subjected to interobserver variability limiting the reproducibility of the method [2, 5]. In order to overcome such limitations, recent interests have been directed towards the development of computer-aided-diagnosis (CAD) systems supporting IIF diagnostic procedure. Such works focus on image acquisition [8, 20], image segmentation [10, 9, 16] and fluorescence intensity classification [14] as well as staining pattern recognition [6, 7, 13, 16, 19].

It is worth noting that none of these papers discusses how to recognise the presence of mitotic cells in the image under consideration. Since the development of CAD system in IIF cannot prescind from the recognition of mitotic cells, in the following we present different features and classification architectures addressing the issue of mitotic cells recognition. Our proposal has been evaluated on an annotated dataset of mitotic cells on which we carried out several tests.

2 Background and Motivations

2.1 Medical context

Current guidelines for ANA tests recommend the use of HEp-2 substrate diluted at 1:80 titer and require to classify both fluorescence intensity and staining pattern. This is a challenging task affecting the reliability of IIF diagnosis [2, 5]. With regard to the classification of fluorescent intensity, the guidelines suggest scoring it semi-quantitatively and independently by two physicians experts of IIF. The scoring ranges from 0 up to 4+ relative to the intensity of
Mitosis is the process by which a eukaryotic cell separates the chromosomes in its cell nucleus into two identical sets in two nuclei.

Positive HEp-2 samples may reveal different patterns of fluorescent staining that are relevant to diagnostic purposes (Figure 2). Although more than thirty different nuclear and cytoplasmic patterns could be identified [21], in the literature they are classified into one of the following groups [19]: (i) **homogeneous**: diffuse staining of the interphase nuclei and staining of the chromatin of mitotic cells; (ii) **peripheral nuclear**: solid staining, primarily around the outer region of the nucleus, with weaker staining toward the center of the nucleus; (iii) **speckled**: a fine or coarse granular nuclear staining of the interphase cell nuclei; (iv) **nucleolar**: large coarse speckled staining within the nucleus, less than six in number per cell; (v) **cytoplasmic**: fine fluorescent fibres running the length of the cell; it is frequently associated with other autoantibodies to give a mixed pattern; (vi) **centromere**: several discrete speckles (∼40–60) distributed throughout the interphase nuclei and characteristically found in the condensed nuclear chromatin during mitosis as a bar of closely associated speckles. Sometimes two concomitant staining patterns can be observed in the same well. In these cases, further dilution and/or better focusing may help to recognize different overlapping staining.

Since IIF is a subjective, semi-quantitative method, physicians act differently when the same sample is presented to them: some are more conservative and others more liberal, depending mostly on their skills and background, giving rise to classification variability. Another significant reason of uncertainty is the low contrast of borderline and negative samples. In order to guarantee the correctness of the test, producers add to the slides some mitotic cells which give to medical doctors the confidence with their decisions.

Mitotic cells are important in IIF for two reasons. First, we can be sure that the well has been correctly prepared if we are able to detect at least one fluorescent mitotic cell. Second, they provide information on the staining pattern since they match with certain kind of patterns, including all stainings of antigens with different distribution throughout the cell cycle, such as midbody, CENP-F, mitotic spindle, centriole/centrosome and NuMA staining [19]. Notice that producers typically guarantee that a certain percentage of mitosis appear in each slide, but give no information about their location.

Mitotic cells of HEp-2 substrate may exhibit two fluorescent patterns. In the first, the cell body is fluorescent, while the collapsed chromosomes mass located in the middle part of the cell does not exhibit a fluorescent pattern, or it has a weak fluorescence. This kind of mitotic pattern is named as **negative mitosis** (panels A and B of Figure 2). In the second pattern, we can observe the opposite situation, i.e. the cell body is weakly or not fluorescent, while the chromosomes mass is fluorescent. An example of this type of mitotic cell, referred to as **positive mitosis**, is shown in panels C and D of Figure 2. In both cases it is worth observing that collapsed chromosomes mass has a circular or elliptic shape, approximately.

### 2.2 Motivations

In IIF, the availability of accurately performed and correctly reported laboratory determinations is crucial for the clinicians. The relevance of the issue is emphasized by the increase in the incidence of autoimmune diseases observed over the last years, partly attributable to both improved diagnostic capabilities and growing awareness of this clinical problem in general medicine. However, the major disadvantages of IIF are: (i) the lack of resources and adequately trained personnel [6, 18]; (ii) the low level of standardization [17]; (iii) the inter and intralaboratory variance [2, 6]; (iv) the lack of automatized procedures; (v) the photobleaching effect, which bleaches significantly in a few seconds biological tissues stained with fluorescent dyes [22].
To date, the highest level of automation in IIF tests is the preparation of slides with robotic devices performing dilution, dispensation and washing operations [1, 3]. Being able to automatically determine the presence of autoantibodies in IIF would enable easier, faster and more reliable tests. Hence, an evident medical demand is the development of a CAD system, which may support physician’s decision and overcome current method limitations, improving the standardisation level and reducing the variability observed within the same laboratory or different laboratories [2]. Recent research has developed CAD tools both for image acquisition [8, 20] and classification [7, 6, 9, 10, 13, 14, 16, 19]. Most of such works recognize fluorescence intensity and/or staining patterns using information belonging to single cells in the image [6, 7, 13, 16, 19], applying image segmentation algorithms based on Otsu’s or watershed algorithms [12] in conjunction with morphological processing [6, 9, 10, 13, 16].

However, a deep analysis of such works reveal that none of them proposed any method for mitotic cells recognition, although some papers have presented approaches tailored to staining pattern classification of single cell. It is worth noticing the ability to automatically detect mitotic cells would permit to develop systems for automatic slide analysis in IIF, from image acquisition up to image classification. Indeed, the presence of these cells is a key factor used by medical doctors to assess, on the one hand, the correctness of slide preparation process and, on the other hand, the reported staining pattern, as described in section 2.1. Therefore, the detection of such cells can help IIF acquisition phase of a CAD system since, for instance, the system should scan the well under examination, look for mitotic cells and then acquire for classification purpose those parts of well containing mitotic cells. Moreover, if mitotic cells are absent, such a system should warn the specialist that something was wrong during the preparation of the slide. With reference to the classification step of a CAD system, the recognition of mitotic cells should reinforce the analysis of staining pattern.

On this basis, this paper proposes a novel approach to recognise mitotic cells thus addressing this lack found in the literature and opening the chance to develop automatic system for IIF image analysis.

3. Features extraction and selection

This section describes how we have selected the heterogeneous set of descriptors used to represent the peculiarities of mitotic cells. They are morphological descriptors inspired by the peculiarities of cells at hand and texture measures, e.g. first and second order histograms, rectangle features and local binary pattern (LBPs).

The first set of features is composed by morphological descriptors, which are based on the observation that mitotic cells may be fluorescent inside or outside the chromosomes mass (section 2.1). We compute features that, on the one side, look for elliptic shape and, on the other side, analyse the fluorescence intensity inside the cells. In order to catch information on the elliptic shape of chromosome mass, being either a positive or negative mitosis, we fit inside the cell body the following model representing a multivariate Gaussian distribution:

$$z = a + m \cdot \exp \left[ - \left( \frac{x - \mu_x}{\sigma_x} \right)^2 - \left( \frac{y - \mu_y}{\sigma_y} \right)^2 \right]$$ (1)

where \(a\) is an offset, \(m\) is a scale factor, \(\mu_x\) and \(\mu_y\) are the mean values of the distribution along the \(x\) and \(y\) axes, respectively, \(\sigma_x\) and \(\sigma_y\) are \(x\) and \(y\) standard deviations, respectively. We also measure the coefficient of determination \(r^2\). Since the chromosomes mass has a particular orientation inside the cells and the cells are randomly oriented in the well, we perform several fittings, each one rotating the cell of 10 degrees. Such a rotation should permit to achieve the best fit of the model to the biological area of interest. For each fitting, the features set is composed of the following parameters: \(a, m, \mu_x, \sigma_x, \mu_y, \sigma_y\) and \(r^2\). It is worth noting that some orientations have a good fitting, whereas previous and next orientations return very low fitting determinations. Furthermore, in these cases we notice large variation between the estimated parameters of multivariate Gaussian distribution. These situations may correspond to false mitosis; indeed, in case of true mitosis, we notice that the fitting determinations are high for several consecutive orientations as well as the parameters values are not so different. On this basis, we derive other sets of features based on the mean values of model parameters measured on consecutive orientations given by 20, 40, 60 and 80 degrees.

In order to catch information on fluorescent intensity inside the cell body, we define other two morphological descriptors as follows. For each cell, we apply the Otsu’s algorithm [12] and then found the number of connected regions whose mean value of fluorescence intensity is larger than Otsu’s threshold. Indeed, in mitotic cell we should
find only one region, either the chromosome mass in case of positive mitosis or the other part of cell body in case of negative mitosis. Cells with other patterns should have more than one region. For instance, cells with nucleolar pattern, which have large coarse speckled staining within the nucleus, should have at least two regions with a fluorescence intensity larger than Otsu’s threshold. Furthermore, we compute also the fraction of cell area with a fluorescence intensity larger than Otsu’s threshold.

The second set of features consists of texture measures that have been successfully used in previous works on HEp-2 classification [13, 14]. They are related to statistical and spectral measures. The former have been extracted both from intensity histogram and from grey level co-occurrence matrix by means of computing their statistical moments, e.g. skewness, kurtosis, energy, entropy, to name a few. The latter have been computed from Fourier transform (FT), Wavelet transform and Zernike moments.

We also computed rectangle features, which are simple descriptors very effective in the field of face detection [15]. Indeed, similarly to face detection, we are interested in detecting regions inside an object. A rectangle feature is computed as a weighted sum of pixels inside adjacent rectangles in the image. Defining as $\text{RecSum}_i(r)$ the weighted sum of pixels into a rectangular region $r$ according to the mask $\omega_i$ [15], rectangle features $\text{RecFeat}$ are given by

$$\text{RecFeat} = \sum_{i} \text{RecSum}_i(r)$$

(2)

Rectangle features owe their success, besides their effectiveness, to the possibility of being efficiently implemented through an intermediate representation of the image called integral image.

LBPs assign to each pixel of the image a label obtained comparing it with its neighbourhood matrix. Different types of LBP operators can be computed varying both the pixel arrangement and the number of neighbours, respectively. In this work we use the standard LBP operator obtained applying a 3x3 neighbourhood square matrix [23]. More specifically, defining as $g_c$ the value of the pixel with coordinates $(x_c, y_c)$, and with $g_p$ the value of its $p$-th neighbour, the LBP operator is defined as follows:

$$LBP(x_c, y_c) = \sum_{p=0}^{7} 2^p s(g_p - g_c)$$

(3)

where

$$s(x) = \begin{cases} 
1 & \text{if } x \geq 0 \\
0 & \text{otherwise} 
\end{cases}$$

The basic LBP operator is neither robust to changes in spatial resolution nor to texture rotations. For this reason we extract also the circular LBP, which is computed using bilinear interpolated circular neighbourhoods, since they demonstrated robustness to grayscale variations and to rotated textures [24].

The search of the best discriminant subset has been performed running greedy stepwise and best first searches, both forward and backward, and using cross-validation. Then, it has been refined by an exhaustive search, taking into account the dimensionality of the dataset and of the features set. At the end of this process we individuated 15 descriptors belonging to morphological descriptors, statistical moments of intensity histogram and grey level co-occurrence matrix, and circular LBP (table 1). Morphological descriptors are: mean values of multivariate Gaussian distribution averaging out consecutive orientations given by 60 degrees, the standard deviations and the fraction of cell area with a fluorescent intensity larger than Otsu’s threshold. From the intensity histogram we selected the mean and the skewness: the former is a measure of average intensity, whereas the letter estimates the asymmetry of the histogram. From the grey level co-occurrence matrix describing image texture we selected the covariance and the inertia, which specify the energy spread about the matrix diagonal. Circular LBP features describe image texture with reference to circular information. In this case we selected statistical measures from the derived second order histograms such as autocorrelation, covariance, energy around the absolute maximum.

4. Experimental evaluation

Since, to our knowledge, there are not reference databases of IIF images publicly available, we populated a database of annotated mitotic cells, using slides of HEp-2 substrate at the fixed dilution of 1:80, as recommended by the guidelines [2]. Specialists take HEp-2 images with an acquisition unit consisting of the fluorescence microscope (40-fold magnification) coupled with a 50 W mercury vapour lamp and with a digital camera. The camera has a CCD with squared pixel of equal side to 6.45 μm. The images have a resolution of 1388x1038 pixels, a colour depth of 24 bits and they are stored in bitmap format. Specialists manually segment and annotate each cell at a workstation monitor since at the fluorescence microscope is not possible to observe one cell at a time, and report data on fluorescence intensity, pattern and mitosis phase. The used database consists of 126 cells, 63 mitotic cells and 63 no mitotic cells, which therefore exhibit one of staining patterns reported in section 2.1. Dataset is available at http://mivia.unisa.it/databases/db_database/biomedical/.

We investigate the performance that could be achieved in mitotic cell recognition when popular classifiers belonging to different paradigms are used. In this respect, we
Table 1. Selected features.

<table>
<thead>
<tr>
<th>Category of features</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphological descriptors</td>
<td>Mean of multivariate Gaussian distribution averaging out consecutive orientations at 90 degrees, standard deviations and the fraction of cell area with a fluorescent intensity larger than Otsu’s threshold</td>
</tr>
<tr>
<td>Intensity histogram</td>
<td>Mean, skewness</td>
</tr>
<tr>
<td>Grey-level co-occurrence matrix</td>
<td>Covariance, inertia</td>
</tr>
<tr>
<td>Circular LBP</td>
<td>Autocorrelation, covariance, energy around the absolute maximum of the output image second-order histogram</td>
</tr>
</tbody>
</table>

test a Multi-Layer Perceptron (MLP) as a neural network, a Naive Bayes classifier as a bayesian classifier, a $k$-Nearest Neighbour as a statistical classifier ($k$NN), a Support Vector Machine (SVM) as a kernel machine, and AdaBoost as an ensemble of classifiers. In the following we briefly report the experimental set-up of each classification architecture:

**MLP** We use a MLP with one hidden layer. The number of neurons in the input layer is given by the number of the features, whereas the number of neurons in the output layer is equal to two. Several preliminary tests have been carried out to determine the best configuration of the MLP in terms of number of neurons in the hidden layer: specifically, configurations from 1 up to 100 neurons were tested.

**Naive Bayes** The Naive Bayes does not demand for specific set-up.

**$k$NN** We explore different values of $k$ in the interval [1; 9] to determine the best configuration.

**SVM** In the SVM experiments, we use a Radial Basis Function kernel. We perform a grid search to determine the best setup of the SVM in terms of Gaussian width, $\sigma$, and of regularisation parameter, $C$. The searches of $\sigma$ and $C$ have been carried out in the intervals [0.01; 30] and [0.1; 60], respectively.

**AdaBoost** For AdaBoost we use decision stumps for base hypotheses, exploring different numbers of iterations (in the interval [10; 100]) to determine the best configuration.

Tests were performed using leave-one-out (LOO) approach, using the training set for the parameters selection phase reported above. Afterwards we report only the results achieved using the best classifier configuration, averaging over the different runs.

To estimate the recognition performance we measure the following parameters: global accuracy ($\text{Acc}$), true positive rate or sensitivity ($\text{Sens}$), true negative rate or specificity ($\text{Spec}$), precision, and F-measure. We also draw ROC curves and use the area-under-curve (AUC) for cell classifiers comparison [4]. Table 2 show the mean values of performance of the five classification paradigms estimated using the LOO approach, where the values of the parameters for the best configuration of each classifier are given in parentheses. In case of MLP the number represents the neurons in the hidden layers, in case of $k$-NN the number is the value of $k$, in case of SVM the values are $\sigma$ and $C$, and in case of AdaBoost the value corresponds the number of iterations. Figure 3 shows the corresponding ROC curves, built using the best configuration of each classifier.

The results show that both $k$-NN and AdaBoost provides the largest performance. While the former achieves the largest accuracy (86.5%), the latter has the best value AUC and the largest sensitivity, whose value is important for the needs of real applications. These results are promising and should open the way to the development of a comprehensive CAD in IIF, since the ability to recognise mitotic cells is important both for image acquisition and staining pattern recognition.
5. Conclusions

In this paper we have presented an approach for mitotic cells classification in HEp-2 slides. Despite recent research has focused on CAD system in IIF, classification of mitotic cells is an issues never discussed in the literature up to now. Such cells play a key role in IIF image classification since specialists look at them to be confident with their decisions. Being able to recognise such cells would permit to assess the correctness of slide preparation process and the reported staining pattern. We have investigate an heterogeneous set of features to represent the peculiarities of mitotic cells and tested five different classifiers achieving promising results. Future works are directed towards the test of this approach on a larger dataset and then the integration of the system for mitotic cells recognition with systems for IIF image acquisition and staining pattern classification. The goal is a comprehensive CAD supporting all phases of IIF diagnosis.

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