AUTOMATED ANALYSIS OF MULTI-SPECTRAL M-FISH IMAGES FOR BREAST CARCINOMA STAGING

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ABSTRACT
M-FISH images are difficult to interpret because the emission spectra of fluorochrome marked DNA probes overlap with each other and with the tissue’s intrinsic auto-fluorescence. Multi-spectral imaging is used to take spatially and spectrally resolved images of M-FISH samples and spectral unmixing is applied to resolve the crosstalk in the emission spectra. The unmixing is realized by a combination of: Alternating Least Squares (ALS) and VARI-MAX. Spot counting algorithms use the high contrast false color images to automatically determine the HER-2/neu to CEP 17 ratio.

KEY WORDS
M-FISH, spectral unmixing, ALS, VARI-MAX, spot counting

1 Introduction

One out of four carcinoma cases among women are diagnosed as breast carcinoma. This makes breast carcinoma the most common cancer form among women. Therefore a reliable diagnosis of breast carcinoma during routine checks is required. The gold standard for staging breast carcinoma is the cytogenic technology called fluorescent in-situ hybridisation (FISH). The technology marks cell components (e.g. proteins, cytoplasm, nucleus) as well as specific DNA sequences with fluorescent markers. Fluorochromes are chemicals that absorb light at a specific energy level and emit light at a lower energy level. Using special filter combinations light from the excitation light source can be separated from the specific fluorescence signal of the DNA markers.

Breast carcinoma samples that are marked with multiple DNA probes are called multi-color FISH (M-FISH). M-FISH has two main problems: first, the broad-band emission spectra of the DNA probes overlap and cannot be resolved even with ideal fluorescence filters. Second, skin components such as elastin or collagen have an intrinsic auto-fluorescence that contains no distinctive spectral features but which degrades image quality. Figure 1 shows typical emission spectra of the fluorochromes: DAPI, FITC, SpectrumOrange and the non-specific emission spectrum of tissue auto-fluorescence (low intensity yellow curve). One can see that even ideal filters (shown as rectangles) are not able to resolve the crosstalk between the different fluorochromes.

Currently, pathologists work with color images of M-FISH samples acquired with a combination of a standard RGB camera and fluorescence filters. However, due to the poor image quality of standard images a reliable diagnosis requires much experience.

In previous work [1, 2] we showed that spectral unmixing methods are able to enhance the contrast of M-FISH images and resolve the spectral overlap in the emission spectra of the fluorescently labeled DNA probes. In this paper we present a complete system that acquires multi-spectral M-FISH images and an algorithm that enhances image quality with spectral unmixing (SU) methods and provides additional information to support a physician with his or her diagnosis. The algorithm works in two stages: First, the 2 SU methods: Alternating Least Squares (ALS) and VARI-MAX, enhance image contrast and resolve the spectral overlap in the emission spectra of M-FISH samples. Then spot counting algorithms use the high contrast false color images to automatically determine the ratio of the human epidermal growth factor receptor 2 (HER-2/neu) to chromosome 17 centroid (CEP 17) [2]. The HER-2/neu to CEP 17 ratio indicates whether the tissue is cancerous.

2 Epidemiology of Breast Carcinoma

Breast carcinoma is caused by uncontrolled cell growth due to a malfunction in the cellular mechanisms [3]. HER-2/neu genes are responsible for the growth and differentiation of cells. Physicians use the HER-2/neu status to...
diagnose breast carcinoma [3]. An increase in the number of HER-2/neu receptors relative to healthy breast epithelial cells, due to gene-amplification, is an early indicator of breast carcinoma [4], [5], [6]. This increase in the number of HER-2/neu spots is shown by more than two fluorescent spots occurring within a cell nuclei.

2.1 Fluorescent in-situ hybridisation (FISH)

HER-2/neu gene-amplification, deletions and translocations in tumor tissue is normally detected using M-FISH. [7]. The technique uses commercially available DNA probes, which are conjugated with a fluorochrome. The DNA probes are hybridized to a specific DNA sequence, which gives the red, green and blue fluorescent spots in the microscopic images when the samples are imaged with special fluorescent filter kits.

2.2 Detection of breast carcinoma

Tissue samples are imaged with fluorescence microscopes, which are equipped with fluorescence filter combinations (e.g. DAPI, FITC and SpectrumOrange). The fluorescence emission peaks of HER-2/neu, CEP 17 spots and the cell nucleus are filtered with the excitation/emission filter combination.

The fluorescent spots of CEP 17 and HER-2/neu are important for this application. Currently medical experts have to count the number of spots of 20 morphologically intact, non-overlapping tumor cell nuclei each of which must have a clear fluorescence signal spot [8]. All cell nuclei need to have at least one CEP 17 and one HER-2/neu signal spot to be counted. Two signal spots of the same size must have a distance of at least the single fluorescence spot diameter to be counted as two signal spots [9], compare Figure 2. Lymphocytes, granulocytes, macrophages, fibroblasts, epithelial cells, signals with low intensity, as well as tumor cells with no clear border or a high background signal need to be excluded from the analysis [9].

3 Automated Spot Counting

Our spot counting algorithm presented in this paper has the following processing steps. First multi-spectral data of M-FISH images is acquired using a custom measurement system. After the data pre-processing the cell nuclei are segmented and spectral unmixing is applied to the data. The high-quality images are then used to segment HER-2/neu and CEP 17 signals and the fluorescent spots are automatically counted to calculate the HER-2/neu to CEP 17 ratio.

3.1 Multi-spectral data acquisition

Eight multi-spectral M-FISH data sets, each consisting of 64 single images with a resolution of 1004 × 1002 pixels were acquired using an Axio Imager M1m (Carl Zeiss, Germany) fluorescence microscope. A liquid crystal tuneable filter (LCTF) (Varispec™, CRI, MA, USA) selected the spectral bands from 400nm to 720nm with a step width of 7nm. A special metal halide lamp X-Cite 120 PC (EXFO®, Canada) was used to excite the samples. As fluorescent signals have weak intensities a deep cooled 14 bit EMCCD camera iXon (Andor, Ireland) was used to acquire the images. Our measurement setup is shown in Figure 3.
varies with wavelength we adjusted the focus for the specific emission wavelength ranges (400nm-500nm, 500nm-600nm, 600nm-720nm) of the fluorescent marked DNA probes. Figure 5(a) shows a false color M-FISH image of a breast carcinoma sample from the wavelengths 475nm, 535nm and 620nm from multi-spectral image data. Pathologists use such standard color images routinely for their diagnosis.

3.2 Data pre-processing

A dark image—obtained by closing the camera aperture; and a white reference—obtained by imaging a teflon white standard, were used for the calibration. This calibration model corrects the spatial and spectral characteristics of the measurement setup with a linear model. For the following processing steps the manually selected region of interest (ROI) was defined.

3.3 Segmentation of the cell nuclei

We use wavelengths between 440nm-470nm to segment the cell nuclei. The intensity values of all channels at each spatial position are normalized using the white and dark standard. The mean spectral image is calculated as follows:

\[ \bar{I}(x, y) = \frac{1}{n} \cdot \sum_{i=1}^{n} I_i(x, y), \] (1)

where \( I(x, y) \) is an intensity image with the two spatial coordinates \( x \) and \( y \), \( n \) is the number of summed up spectral images.

We threshold \( \bar{I}(x, y) \) to obtain a binary image. Morphological operations are then applied to fill holes within the nuclei; to remove single pixels from the borders of the nuclei; and to remove cell nuclei that are too small. A distance transform, using the Euclidean distance, is then applied to the binary image to resolve single fluorescent spots. To avoid over-segmentation by subsequent processing the distance image is then median filtered.

Finally, the watershed algorithm is used to separate the nuclei from the background and from each other. Figure 5(a) shows an example with the segmented cell nuclei overlaid onto the spectral image. Nuclei at the borders of the image and also nuclei without fluorescent HER-2/neu spots or CEP 17 spots were automatically removed from the classification result.

3.4 Spectral Unmixing

We use the two SU methods: Alternating Least Squares (ALS) and VARIMAX to reduce the effects of noise and auto-fluorescence. By applying SU to the data we optimize the classification of the HER-2/neu spots and the CEP 17 spots. All of the described algorithms are semi-supervised.

Although the unmixing (i.e. the determination of pure reference spectra) is unsupervised the number of desired components has to be specified by the user.

3.4.1 VARIMAX

VARIMAX [10], [11], [1] extracts reference spectra (material fingerprints), from a data set. The method retains the user defined number of eigenvectors (loadings) \( V \) by a Principal Component Analysis (PCA). Loadings are often called abstract factors, because they are usually completely different from the real factors pure spectra. The value \( s \) called ‘simplicity’ is used to estimate how close this particular factor is to the pure spectrum. Simplicity \( s_i \) of the i-th spectrum is calculated by

\[ s_i = \frac{1}{N} \sum_{k=1}^{N} \frac{X_i^k - Y_{1,i}^k}{N}. \] (2)

The loading matrix \( V \) is then iteratively planar rotated by

\[ X' = VX. \] (3)

This step tries to maximize simplicity to calculate the abstract loadings. \( X \) is a \( M \times N \) matrix with \( M \) rows and \( N \) columns. Each row contains one spectrum and each column represents a certain wavelength. The rows of \( X \) are denoted by \( x_i^T = (x_{i,1}x_{i,2}...x_{i,N}) \) and represent distinct spectra. To guarantee a global maximum the optimization algorithm has to be repeated several times.

3.4.2 Alternating least squares (ALS)

Alternating least squares (ALS) is also a purification algorithm. It assumes that spectral values and abundances are positive, and that a set of pure candidate spectra \( Y_0 \) can be obtained e.g. by Orthogonal Projection Analysis (OPA) [12]. If they were definitely pure spectra then abundances could be found by solving the least squares problem

\[ X = Z_1 Y_0. \] (4)

\( Z_1 \) is the pure concentration of a component and is calculated by

\[ Z_1 = XY_0^T(Y_0 Y_0^T)^{-1}. \] (5)

However, in practice one has to deal with imperfect candidate spectra. Simple factors are orthogonal and usually contain positive and negative values, leading to the situation that some abundances obtained by the least squares solution will be almost for sure negative. In ALS they are clipped to zero \( Z_{1,c} \) and used again to recompute spectra candidates with Eqn. (5).

\( X \) can also contain negative spectral values. With
\[ X = Z_{1,c}Y_1 \]  

and

\[ Y_1 = (Z_{1,c}Z_{1,c})^{-1}Z_{1,c}X. \]  

they are clipped to zero resulting in \( Y_{1,c} \). The algorithm is iterated until the convergence criterion

\[ dY_1 = Y_{1,c} - Y_0 = \frac{tr(dY_1^T dY_1)}{tr(Y_{1,c} Y_{1,c})} < \epsilon. \]

is met. 'tr' stands for trace. It is the sum of the elements on the main diagonal of the determinant. If the algorithm converged or the number of defined iterations is reached then \( Y \) is set to \( Y_{1,c} \) and \( Z \) is set to \( Z_{1,c} \). Otherwise \( Y_0 \) is set to \( Y_{1,c} \) and iterations go on.

### 3.5 Spectral unmixing results

The SU algorithms were used to reduce tissue auto-fluorescence and enhance image contrast. For our analysis we combined VARIMAX and ALS to unmix multi-spectral M-FISH images.

#### 3.5.1 Reduction of tissue auto-fluorescence

Tissue auto-fluorescence degrades image quality. It has an emission spectrum that does not have any distinctive spectral features.

For the analysis we calculated the the mean tissue auto-fluorescence of a given region of interest (ROI) \( N \)

\[
\overline{AF}_N(\lambda_N) = \frac{1}{|N|} \sum_{(x,y) \in N} I(x,y,\lambda_N), \quad (x,y) \in N
\]

Equation 9 takes every pixel \((x,y)\) in a given ROI \( N \) and calculates the mean intensity for each channel \( \lambda_o \) of the spectral image. The result of the SU combination is compared with standard color images that were generated by summing up all images in the specific wavelength range (blue channel: 450nm to 480nm; green channel: 515nm and 555nm; red channel: 595nm and 660nm). It has been shown by [1] that compared to color images the combination of VARIMAX and ALS reduces tissue auto-fluorescence by:

- 80% for HER-2/neu spots,
- 64% for CEP 17 spots
- 90% for cell nuclei spots

#### 3.5.2 Enhancement of image contrast

The broad-band emission spectra of the DNA probes overlap and cannot be resolved even with ideal fluorescence emission filters. This is why we cannot distinguish between pixels that belong to cell nuclei, CEP 17 genes, HER-2/neu genes or tissue auto-fluorescence. Figure 4 (left) shows a 3D plot of a standard RGB image—there is a large overlap of the four point clouds (cell nuclei, CEP 17, HER-2/neu, background). Fig 4 (right) shows that the combination VARIMAX/ALS resolves the point clouds more effectively.

To assess the enhancement of image contrast, a standard RGB image was compared with the results of the SU method. For the evaluation we thresholded (0.5 for cell nuclei, 0.3 for CEP 17 and HER-2/neu) the image and counted the pixels that were ambiguously assigned as cell nuclei, CEP 17 or HER-2/neu pixels. We showed that in a standard RGB image 21.8% of all pixels could not be assigned unambiguously. The combination VARIMAX/ALS reduced this value to 1.1% [1].
3.6 Segmentation of CEP 17 spots and HER-2/neu spots

After the spectral unmixing, the image containing the HER-2/neu spots and the image containing the CEP 17 spots were converted to binary images by applying a threshold. Morphological operations filled holes inside the areas of the HER-2/neu and the CEP 17 spots and removed single pixels from the borders of the spots and regions which are too small. In the last step the spots were labeled for the following counting algorithm. In Figure 5 (a) the reconstructed RGB image is shown, (b) shows the segmentation result for the cell, (c) shows the segmentation result for the HER-2/neu spots and (d) shows the segmentation result for the CEP 17 spots. Figure 6 shows the combined classification results for the cell nuclei, HER-2/neu spots and the CEP 17 spots. The cell nuclei borders are marked by a blue line.

3.7 Determination of the HER-2/neu to CEP 17 ratio

The recommended method [8] to determine the HER-2/neu to CEP 17 ratio is to select 20 nuclei and then to divide the number of HER-2/neu spots by the number of CEP 17 spots. If the ratio is smaller 2 HER-2/neu gene amplification was not observed, if the ratio is greater 2 gene amplification is observed by FDA and a therapy is needed. A ratio smaller than 3 is called low gene amplification. If there are more than 3 CEP 17 signals within a nucleus a polysemy is detected. A HER-2/neu to CEP 17 ratio between 1.8 - 2.2 is defined as borderline. If there are 4.0 - 6.0 HER-2/neu spots within one nucleus this is also classed as borderline. In this case 20 additional nuclei have to be counted and the ratio has to be recalculated [8]. The HER-2/neu to CEP 17 ratio was calculated from the mean HER-2/neu to CEP 17 ratio for the 11 nuclei in the example data-set. Because the CEP 17 spots do not form spatial clusters, the counting of the CEP 17 spots was directly performed on the segmentation result shown in Figure 5 (d). To estimate the number of HER-2 spots per nuclei we divide the area of the nucleus (in pixels) by the average size of the HER-2/neu spot.

Table 1: Spot counting results for the example data-set.

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<tr>
<th>Nucleus ID</th>
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<th>Number of HER-2/neu spots</th>
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4 Conclusion

Spectral unmixing methods have been applied to multi-spectral M-FISH image data of breast carcinoma tissue samples. It is shown that SU is a powerful tool to reduce non-specific tissue auto-fluorescence and to resolve crosstalk in the emission spectra of fluorescently labeled DNA probes. This enhancement of M-FISH images is an important pre-processing step to enable automated spot counting algorithms. This paper has shown that the two techniques can be combined to produce an integrated system that aids the physician in his diagnosis.
The current system is still in the prototype phase and needs to be tested on more data sets. Future work will include a wider data set, including borderline M-FISH samples with a HER-2/neu to CEP 17 ratio between 1.8 and 2.2. Another technique called D-ISH (Dual in-situ hybridisation, Ventana, Roche), which slightly differs in the sample preparation and the counting criteria will be included to the analysis. Both, M-FISH and D-ISH results will be compared with the spot counting results of an experienced pathologist.

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References


