Isolation and measurement of the features of arrays of cell aggregates formed by dielectrophoresis using the user-specified Multi Regions Masking (MRM) technique

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Abstract. The use of dielectrophoresis for the construction of artificial skin tissue with skin cells in follicle-like 3D cell aggregates in well-defined patterns is demonstrated. To analyse the patterns produced and to study their development after their formation a Virtual Instrument (VI) system was developed using the LabVIEW IMAQ Vision Development Module. A series of programming functions (algorithms) was used to isolate the features on the image (in our case; the patterned aggregates) and separate them from all other unwanted regions on the image. The image was subsequently converted into a binary version, covering only the desired microarray regions which could then be analysed by computer for automatic object measurements. The analysis utilized the simple and easy-to-use User-Specified Multi-Regions Masking (MRM) technique, which allows one to concentrate the analysis on the desired regions specified in the mask. This simplified the algorithms for the analysis of images of cell arrays having similar geometrical properties. By having a collection of scripts containing masks of different patterns, it was possible to quickly and efficiently develop sets of custom virtual instruments for the offline or online analysis of images of cell arrays in the database.

1. Introduction
During embryonic development of skin tissue in chicken feather bud primordia are formed in regular hexagonal patterns. Patterning starts at a line above the neural tube, which subsequently breaks up into individual placodes. Formation of placodes then spreads from this line in a cascade over the whole skin, forming a hexagonal array of feather buds. The exact details of the mechanisms by which this pattern is formed are still unknown, but are thought to involve the condensation of cells into aggregates (placodes) whose position is determined by a reaction-diffusion mechanism of activator and inhibitor molecules distributing themselves at differential rate across the skin [1].

The creation of artificial skin with cell aggregates of known size and shape spaced in well defined and regular patterns could help with elucidating the mechanisms by which the regular arrays of feather bud primordia are formed. In this study the use of dielectrophoresis (DEP), the lateral movement of particles in non-uniform electric fields [2] was explored for the formation of cell aggregates of controlled size and shape in well-defined patterns.
After the formation of the aggregates it is often necessary to analyze the patterns produced and to study their development after their formation. To aid in this we have developed LabVIEW-based Virtual Instrument (VI) system to effectively and simultaneously analyze images of microarrays of cell aggregates using IMAQ Vision Development Module, which we will describe here.

2. Methods
Electrode designs were made using Protel Design Explorer 99. Electric field calculations were made using COMSOL Multiphysics. Microelectrodes were made from ITO-covered glass slides using standard photolithography. A simple microfluidic chamber was created above the micro electrode array using glass slides and a silicone spacer (see Figure 1). Chicken embryonic skin cells were isolated by enzymatic tissue digestion from the dorsal part of an 8-day old white leghorn chicken embryo. The dissociated skin cells were suspended in Dulbecco medium (DMEM) prior to DEP experiments. When needed, the cells were centrifuged and washed several times before transferring to low conductivity iso-osmotic sorbitol solution (300 mM, $\sigma = 6.1 \times 10^{-4} \text{ S m}^{-1}$). The cells were introduced into the DEP chamber (approximate height = 500 $\mu$m) at a constant flow rate of around 40 $\mu$L minute$^{-1}$ using a peristaltic pump.

Figure 1. Dielectrophoresis chamber for the formation of well defined biodielectric patterns.

Construction of the biodielectric patterns involved the application of a high frequency (1 MHz, 10 $V_{pk-pk}$) AC signal using TG120 Thurlby-Thandar frequency generator, to the microelectrodes to create non-uniform electric fields and use dielectrophoresis to force the cells together. This was followed by the immobilization of the cells in a 50% Puramatrix gel in order to create a skin-like material and enable the long-term incubation of the immobilized cells. Images were taken of the aggregates using a widefield stereo microscope from Celsi and a NIKON Coolpix E4500 digital camera. Image analysis was performed with LabVIEW IMAQ Vision.

3. Formation of Various and Defined Biodielectric Patterns
As shown previously [3] the shape of cell aggregates between a set of castellated electrodes can be predicted using standard finite element analysis package such as COMSOL Multiphysics. The shape of the cell aggregates could be expected to follow the pattern of the high electric field strength regions between castellations.
Electric field patterns formed between electrodes of different shapes are shown in Figure 2. 2D images of the actual aggregates of skin cells formed and their associated pseudo-3D representations based on the distribution of the pixel intensities are shown in Figure 3(I) and 3(II) respectively. Although the shape of the cell aggregation followed the predicted pattern based on electric field calculations well, it was affected by factors such as the liquid flow rate and the concentration of cells entering the chamber, as well as the electrode geometry, frequency and potential difference between the castellations.

![Electric field patterns](image)

Figure 2. Modelling of electric field calculation between sets of castellated electrodes with various design patterns (in meter unit). A. Small Hexagon. B. Line Pattern. C. Large Hexagon. D. Elongated Hexagon. E. 'W' Pattern. F. Round Edge with Hexagonal Pattern.
Figure 3. I. Images of embryonic skin cell aggregates formed with DEP in various different patterns. II. 3D representation of cell aggregates between electrode castellation based on pixel intensity. A. Small Hexagon. B. Line Pattern. C. Large Hexagon. D. Elongated Hexagon. E. ‘W’ Pattern. F. Round Edge with Hexagonal Pattern. Scale bar is 100 µm.

4. Analyzing the Images Using User-Specified Multi Regions Masking Technique

Analysis of the images of cell aggregates could provide information about - amongst others - the quality of the aggregates, the aggregation process itself, and the development of the aggregates after their formation. To achieve this, it is necessary to take a series of graphical programming steps to convert the original image to binary version showing only the regions of interest (in our case; cell aggregates in the regions specified by the castellations), based on a certain threshold value (illustrated in Figure 4). Once the correct (representative) binary version of the particles is achieved, subsequent analysis is relatively straightforward.

Figure 4. Diagram illustrating differences in graphical programming steps from original image to the formation of binary version of the image.
A series of graphical programming functions (algorithms) was tested and optimized in order to develop Virtual Instruments (VIs) which are capable of analyzing images of arrays of aggregates in a database by simply changing the set parameter values according to the unique conditions of the individual images (e.g. light intensity, edge sharpness). Due to the complex processes that occur during DEP pattern formation and the large number of potential sources of interference (such as unbound cells, contaminating cell debris or other particles), the isolation and measurements of distinct features (immobilized cell aggregates) in the image was complicated. This can be clearly seen in Figure 3, where cells can be seen to aggregate in areas outside the main areas of interest as local high electric field regions also occur outside the main areas [4]. The problems were minimized by using the user-specified multi-regions masking technique prior to binarizing the image for subsequent automatic particle measurements. A similar masking approach was previously used by Gascoyne et al. [5] for measuring aggregate formation with dielectrophoresis. However, their interest was mainly in the speed of the particles forming the aggregates, whilst our interest is mainly in the shape, size and position of the aggregates.

It was found that the best method of specifically isolating only the cell aggregates for further analysis involved applying a set of user-specified multi-region masks prior to thresholding the images based on other parameter values. Thus, image analysis using LabVIEW IMAQ Vision mainly involved the following major group of functions [6]:

- **Image Acquisition** (Algorithm used: *Image open*). This step could be performed online / real-time using various types of image acquisition [7] hardware or offline (from large collection of images previously saved in a database). In this work only the offline method of image analysis was used.

- **Pre-Processing**. This step is where multi-regions masking technique was applied (Algorithm used: *Image Masking*). A series of maskings was applied on the displayed image using the various masking tools available in the package (circular / oval, square, polygon, annulus, rotated rectangle, or freehand region tool). This was achieved by holding Ctrl button (in PC) during multi-regions masking. The mask covered or filtered out the majority of the unwanted regions in the image, leaving only the regions of interest for further analysis (see Figure 5-I). Other algorithms that could be used in this pre-processing step are *Calibration* (to give real-life metric measurements instead of pixel measurements), and *Plane Extraction* (to give grayscale version of the original full color image).

- **Thresholding**. This step isolated the pixels of interest in the previously unmasked areas (to be a set value = 1) and set the remaining pixels as background pixels (set value = 0) thereby creating a binary image. A locally adaptive thresholding method (*Niblack*) was used which categorized a pixel as part of particle or the background based on the intensity statistics of its neighboring pixels. This method was particularly good when the original images exhibited non-uniform lighting effect during acquisition.

- **Filtering**. Because thresholding is a subjective process, the resulting binary image may still contain unwanted information, such as small residual noise particles. Use of the Multi-regions masking technique cut out the majority of these noises. The remaining filtering algorithms used were therefore *Remove small objects* and *Remove border objects* (i.e. the mask itself). Other optional algorithms that were sometimes needed fell under binary morphology modification functions. These included *Fill holes, Close objects*, and *Dilate / Erode objects*. The final binary images formed the closest possible representative shapes of the actual biodielectric patterns (Figure 5-II).

- **Measurement**. IMAQ Vision *Particle Analysis* could be used to calculate all (up to 49) requested measurements in pixel or metric values. Measurements used here included object perimeter, area, hydraulic radius, elongation factor and Heywood Circularity factor (Table 1 and Table 2).
The final step was the generation of custom VIs, based on selected algorithms above. This was used to analyze biodielectric images requiring similar mask templates and the same geometrical pattern. The same script could be used for batch processing other images in a library [8]. However, adjustment of the threshold value was often required for a more representative binary version of the original image.

The calculations performed were relatively fast. All steps taken to analyze all objects simultaneously in each image (including isolation and measurement processes) could be implemented efficiently with a total time less than 0.5 seconds, on a 2 GHz Pentium 4 processor.

![Conversion of user-specified masked images to binary large objects](image)

**Figure 5.** Conversion of user-specified masked images (I) to binary large objects (II), a group of contiguous pixels that have the same intensity value (i.e. 1). A. Small Hexagon. B. Line Pattern. C. Large Hexagon. D. Elongated Hexagon. E. ‘W’ Pattern. F. Round Edge with Hexagonal Pattern. Scale bar is for 100 µm.

### 5. Results of Image Analysis and Discussion

The results of the analysis of the images shown in Figures 3 and 5 using the user-specified multi regions masking technique described here are given in Tables 1 and 2.

Table 1 confirms that the masking technique can be used to generate data from images of cell aggregates formed at a variety of electrode shapes. Table 2 shows a comparison of the distances and angles between cell aggregates and compares them with the expected values based on the specifications of the actual photo mask design. The analysis shows a very good agreement between the two sets of data.
Table 1. Summary of 2D geometric measurements / analysis of aggregates on various electrode designs. All electrodes had a characteristic size of 100 µm. Values given are the mean and standard error of mean.

| Microelectrode Designs | Mask Type | Perimeter (µm) | Elongation Factor | Circularity Factor | Area (µm²) | Hydraulic Radius |
|------------------------|-----------|----------------|-------------------|-------------------|------------|-----------------|
| Small Hexagon          | Circular  | 290.1 ± 10.12  | 1.97 ± 0.04       | 1.41 ± 0.03       | 3410.2 ± 197.2 | 11.71 ± 0.42    |
| Line Pattern           | Square    | 4772.2 ± 101.2 | 10.53 ± 0.51      | 2.72 ± 0.08       | 247428.7 ± 14917.6 | 51.85 ± 2.80   |
| Large Hexagon          | Square    | 1119.1 ± 10.3  | 2.43 ± 0.03       | 1.38 ± 0.02       | 52590.7 ± 970.2  | 47.03 ± 1.06    |
| Elongated              | Oval      | 525.2 ± 10.3   | 3.21 ± 0.10       | 1.56 ±0.03        | 9067.2 ± 363.1  | 17.26 ± 0.62    |
| ‘W’ Pattern            | Polygon   | 9320.1 ± 587.9 | N/A               | N/A               | 223079.2±7683.2  | N/A             |
| Round Edge             | Oval      | 495.9 ± 21.8   | 3.38 ± 0.21       | 1.73 ±0.09        | 8993.2 ± 197.4  | 18.62 ± 0.97    |

N/A = Not Available

Table 2. Summary of measured distances and angles between cell aggregates formed at different electrode designs and the expected value based on the electrode design. All electrodes had a characteristic size of 100 µm. Values given are the mean and standard error of mean.

| Microelectrode Designs | Vertical Distances (µm) | Exp. Value (µm) | Horizontal Distances (µm) | Exp. Value (µm) | 3-Points Angle 1 (º) | Exp. Value (º) | 3-Points Angle 2 (º) | Exp. Value (º) |
|------------------------|-------------------------|-----------------|---------------------------|-----------------|----------------------|----------------|----------------------|----------------|
| Small Hexagon          | 393.2 ± 1.2             | 400             | 442.7 ± 1.3              | 450'            | 115.2 ± 1.1          | 116.4          | 124.8 ± 2.3          | 127.3          |
| Line Pattern           | 402.0 ± 5.0             | 400             | N/A                      | N/A             | 180.1 ± 1.1          | 180            | N/A                  | N/A            |
| Large Hexagon          | 397.8 ± 3.6             | 400             | 803.5 ± 5.8              | 800             | 119.1 ± 2.7          | 120            | 122.5 ± 5.1          | 120            |
| Elongated              | 401.7 ± 2.8             | 400             | 799.5 ± 3.9              | 800             | 121.9 ± 4.1          | 120            | 119.7 ± 4.8          | 120            |
| ‘W’ Pattern            | 403.6 ± 6.6             | 400             | N/A                      | N/A             | 181.4 ± 3.7          | 180            | N/A                  | N/A            |
| Round Edge             | 398.4 ± 2.9             | 400             | 807.1 ± 5.9              | 800             | 120.8 ± 1.9          | 120            | 121.3 ± 2.9          | 120            |

Diagonal data shown. Exp. = Expected Value, N/A = Not Available

It should be noted that the multi regions masking technique is invariant to the scale, shape and rotation of the features as the users can specify the region just outside the features of interest. The technique is therefore highly useful in the sense that a single mask design can correctly isolate other features (from other images in the database) within the unmasked area with only a few threshold level adjustments, depending on the variation in illumination level and camera viewpoint during acquisition.

Successful isolation of features is often a key and fundamental aspect of many problems in computer vision and image analysis. The multi regions masking technique described here is particularly useful due to their reusability with only a few threshold level adjustments. Furthermore, statistical analysis can be performed on the data to determine whether the changes are significant or not.
There are many directions for further research in which algorithm development includes addition of recognition feature of biodielectric patterns, based on either shape or in combination with their distinctive color. This includes algorithm development which aimed to individually learn the repeating structure (individual cell aggregate) which will then be used to recognize and subsequently analyze other object within the same group or categories. Some of these developments may combine or utilize the shape recognition technique with edge-based features [9].

6. Conclusions
It has been shown that dielectrophoresis can be used to create arrays of cell aggregates in well-defined patterns. By controlling the design of the microelectrode geometry it is possible to manipulate the 2D and 3D geometry of the resulting cell aggregates. This control includes their spacing (distance and angle between the aggregates), their size and shape as well as cell density distribution. Control of the timing of the introduction of different cells entering the chamber (e.g. dermal cells followed by epidermal cells on top) allows one to form distinct multi-layer micro niches within a controlled microenvironment [10], making it possible to use the system as a tool for the study of the interactions between cells and their microenvironment [11].

It has also been shown that the Multi Regions Masking technique greatly simplify sets of graphical programming (functions) used in image analysis. It allows users to focus directly on the region of interest (ROI) and perform various numerical measurements only on the desired regions (objects). LabVIEW IMAQ Vision Assistant facilitates the development of custom Virtual Instruments for subsequent batch image processing with only a few (minor) threshold level adjustments. The custom VI development is particularly useful if combined with a permanent imaging setup (giving minimum variation in illumination level between acquisition batches). The results of image analysis performed here and the geometry of electrode design used have shown excellent agreement in terms of expected distance, angle and orientation of the desired features.

7. References
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