**Automated Gating of Flow Cytometry Data via Markov Random Fields[[1]](#footnote-1)**

**Running Headline:** Markov Random Field Clustering for FCM Data

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**Introduction**

In an age of ever advancing technology the requirement for automated solutions to analyse the high-dimensional data produced by measurement devices is at the fore-front of statistical research. Flow cytometry (FCM) is one measurement device that has seen a stark improvement in the underlying technology (1, 2). Unfortunately a lack of development in automated analysis tools has restricted the deployment of this state-of-the-art sensor technology (3). The standard for FCM analysis to date has relied heavily on manual expert-driven approaches rather than a unified automated statistical framework (4). The development of such a statistical framework is in strong demand (2, 4-8), and would allow for reproducible and standardised analyses to be conducted, while also reducing the considerable time-investment currently required for manual analysis in FCM.

FCM analysis involves two key stages: the gating stage, where sub-populations of interest are identified and the tagging stage, where correlations between the characteristics of identified sub-populations are explored. The gating procedure currently involves the manual drawing of gates to specify regions of interest in a 2D graphical representation of a pair of FCM variables (7, 10-12). This manual expert-driven approach is highly subjective across laboratories and to a lesser degree across colleagues within individual laboratories. In addition, the projection of high-dimensional data to a 2D graphical representation can lead to substantial information loss (13, 14). This information loss can result in identified cell populations not being representative across all dimensions of the data. A methodology based on reliable statistical inference with appropriate software implementations would aid in reducing the subjectivity and hence variability associated with manual gating currently employed in FCM.

The process of identifying groups of observations which are similar within each group and dissimilar between groups is referred to as clustering in statistics. Gating is an application of statistical clustering where the requirement is to identify cell populations that are homogenous in nature. As such, a variety of statistical clustering techniques have been applied to gating FCM data (9, 15, 16). The k-means algorithm and its extensions have been utilised extensively (8, 17-19), however classical k-means methods only allow cells to belong to one cluster. This hard clustering approach is restrictive in nature and thus a fuzzy k-means approach, allowing cells to belong to multiple clusters with an associated probability of cluster membership, was proposed (19). However, the criterion utilised to select the ‘best’ clustering solution in these approaches can unduly restrict the shape, size and orientation of identified sub-populations, thus allowing for the possibility of failing to identify true clusters. Many authors have attempted to use supervised learning algorithms such as neural networks (20, 21) and support vector machines (22, 23) to automate the gating procedure. However these approaches require the availability of training data which often renders them unsuitable in a field where training data is not always available.

In addition to the application of standard clustering techniques to FCM analysis, model-based clustering has become prominent as an automated gating solution for high-dimensional data (13, 14). Model-based clustering methods assume that sample observations arise from a mixture of one or more probability densities (24-27), where each probability density represents a unique sub-population or cluster. Typically the approach has been to assume that each mixture component follows a *p*-variate Gaussian distribution, with the number of clusters identified via a model selection criterion such as the Bayesian information criterion (BIC) (28). The implications of the Gaussian assumption are that the resulting sub-populations will be elliptical in shape which is not always true for FCM data. Lo et al. (14) considered model-based clustering with *p*-variate mixtures of *t*-distributions in FCM analysis. The larger tail of the *t*-distribution makes the approach more robust to outliers, a common feature of FCM data due to cell debris and doublets, but still retains the elliptical shape constraint of Gaussian mixtures. However in general, the identified sub-populations in FCM do not conform to elliptical clusters even after appropriate data transformation and/or when accommodation is made for outliers.

While several approaches for the automation of FCM gating have been proposed, none to date have utilised the inherent structure of the underlying data. The use of an analogue-to-digital converter (ADC) to process the individual wavelength intensities results in FCM data being discretised. The range of discrete integer values observed in the resulting data is governed by the resolution of the ADC, where a higher resolution allows for considerably more unique integer values to be assigned to varying wavelength intensities (29). While the use of standard statistical clustering tools and model-based clustering, which are designed for continuous data, are not inappropriate in a discrete setting, this paper proposes a methodology that provides a solution for automatic gating by exploiting the unexplored structural layer embedded in FCM data.

The proposed methodology will follow closely the FCM data analysis framework proposed by Bashashati and Brinkman (9). It will be assumed that the quality assessment and normalisation components of the framework will be completed by the experimentalist, and hence the focus will be on the succeeding three components in the pipeline; outlier removal, automated gating and cluster labelling. This paper considers the three aforementioned components as a single overarching component rather than three individual elements of the analysis. As such the methodology outlined in this paper will address all three components by providing a single algorithmic solution which combines methodology from statistical literature and the field of image processing and segmentation.

**Materials and Methods**

***Flow Cytometry Data***

The proposed automated gating solution will be exhibited by using two publicly available FCM datasets, the rituximab data (30) and the Graft-versus-Host-Disease (GvHD) data (31). The use of these two FCM experiments will allow not only a demonstration of proposed methods but permit comparison to previous published solutions to the problem of automated gating where the same data sources have been used (14, 32).

The Rituximab data (30) were collected in an experiment to identify the enhancement of antilymphoma activity of rituximab by different agents. Sixteen hundred varying compounds were distributed to 96-well plates and underwent an initial incubation period. Following this, Rituximab was added to one of the duplicate plates, and both plates were incubated for a further period. The experiment thus contained cells treated with the compound alone, cells treated with compound and Rituximab, and as controls, untreated cells and cells treated with Rituximab alone. Throughout the process cells were stained with BrdU which allowed new synthesized DNA to be labelled. In addition, following the completion of culture the samples were dyed with anti-BrdU and 7-AAD, a DNA binding dye. The cells were analysed using flow cytometric high-content screening recording forward-light scatter (FSC), sideward-light scatter (SSC) and fluorescent markers of anti-BrdU and 7-AAD, resulting in 1545 cells across the four variables.

The GvHD data (31) were collected from stem cell transplant recipients. GvHD is one of the most common complications observed in clinical transplantation of bone marrow, whereby the tissues of the recipient, often the liver and gut, are attacked by donor-immune cells in the graft. The GvHD experiment was initiated to identify the key biomarkers which lead to the development of GvHD in patients. Blood samples from 31 patients were taken and assigned to 96-well plates with 10,000-100,000 cells per well. The well plates were then dyed with 10 different four-colour antibody combinations. Samples from two patients both containing physical property variables FSC and SSC, and four additional fluorescent markers, anti-CD4, anti-CD8β, anti-CD3 and anti-CD8, are considered in this paper. One patient later developed acute GvHD while the second was taken from a control group. Both samples contain the six aforementioned variables measured for greater than 12,000 cells.

***Cytometry in Practice***

In practice the analysis of FCM data is rarely carried out on the entire cell population recorded. Instead an initial partition of the data is produced by gating on FSC and SSC. This initial partition provides an appropriate way of removing outliers and doublets from the analysis as FSC and SSC correspond to physical properties of the cells, namely shape and granularity. Similar to the work of Lo et al. (14) and Hahne et al. (15), we focus on this structured approach to gating throughout this paper. The initial step subsets the recorded observations into two groups, a group which will be used for further analysis and a group which will be considered to be cell debris not relevant to the analysis. However, the proposed gating strategy can be applied to any pair of recorded FCM variables.

***Markov Random Fields Modelling***

Markov random fields (MRF), an *n­*-dimensional random process defined on a lattice grid, emerged from probability theory as a new type of stochastic process, driven by the widespread use of the Ising model (33). Since then MRFs have been used extensively in the field of image processing (34-37) for restoration, removal of noise/outliers, and segmentation, identification of sub-images. Let define a finite lattice grid and specify the first order neighbours of cell such that

which corresponds to the horizontal and vertical neighbours of (36). The lattice with the defined neighbourhood system is thus a MRF if and only if the value depends only on it’s neighbourhood, expressed mathematically as

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The Ising model (38) is a special case of MRFs where the underlying lattice takes ‘binary’ values, with a neighbourhood system . The energy function for the nearest neighbour Ising model with zero external field is defined as

where denotes that the nodes are neighbouring pairs and is the interaction strength. For a decrease in energy occurs when neighbouring cells are in a state of agreeement. Following from this statistical mechanics states that the probability of a given configuration from all possible configurations is defined as

where is the temperature of the system, Boltzmann’s constant is defined as and is a normalising constant such that

***Metropolis Algorithm with Simulated Annealing***

The minimisation of the global energy function of a MRF is catogorised as a combinatorial optimisation problem, whereby the underlying Bayesian methods coupled with Markovian properties result in a non-convex energy function. The Metroplis algorithm (39), introduced in statistical physics, is a Monte-Carlo simulation procedure utilised to determine equilibrium states of thermodynamical processes. The Metropolis criterion relies on the calculation of the change in energy, , from a trial pertubation, , to the current configuration, , such that the trial differs in only one element to the current configuration. If then the is accepeted with probability 1 due to the reduction in global energy. If , that is there has been an increase in the global energy, is accepted with probability while the configuration remains unchanged otherwise (40).

Simulated Annealing (SA) allows for a computationally efficient approach to solving the problem of identifing a global minimum in the global energy function defined on the lattice (41, 42). The approach is to begin with a relatively high initial temperature resulting in a large acceptance probability for changes which induce an increase in the global energy function. The procedure repeats a sequence of Metropolis algorithms until some equilibrium state is achieved. Upon achieving an equilibrium state at the temperature is decreased and a sequence of Metropolis algorithms are iterated until a new equilibrium state is achieved at the new temperature level. The temperature is continuely decreased until the lattice system is frozen.

The SA algorithm requires that be high enough that regardless of the energy change practically all pertubations are accepted. The exact value of depends on values of the unknown energy function undergoing minimistaion, however multiple authors have proposed procedures that provide reasonable estimates (42-44). Throughout this methodology the initial temperature will be set at (44).

The cooling schedule of the process resulting in the decrease of the temperature following an equilibrium state is treated as a fractional decrease of the current temperature such that , will be used throughout this paper (42). Finally, the convergence of the SA approach is defined such that the change in energy across equilibrium states for varying tempertures is close to zero.

***Hierarchial Markov Random Fields***

Hierarchial or multigrid MRF approaches (45-47) are used to extract features of from the data at varying resolutions. In this methodology the hierarchial approach to MRF will be utilised to infer the initial configuration of when beginning the SA approach for a defined resolution. The hierarchial procedure produces a pyramid like structure whereby the coarsest grid contains only a single corresponding to a group of sites at the grid below it, continuining in an expanding fashion to the lattice that defines the field (48). The methodology here does not utilise a complete pyramid structure but instead generates all levels from up to the dimensions of the governing lattice.

***Connected Component Labelling***

Connected component labelling (49) is an established pattern recognition tool used extensively in the identification of disjointed regions in binary, and with adaptations non-binary, images (50-52). Connected component labelling works by traversing an image, pixel-by-pixel, to identify regions of connected pixels based on their intensity values. In the binary setting, utilised in this methodology, the intensity values can be viewed as an active (intensity of 1) or inactive pixel (intensity of 0) in the image. The algorithm traverses the pixels row-by-row until an active pixel is found, the algorithm then proceeds as follows:

1. If all four neighbours of the pixel are inactive, assign a new label to the pixel;
2. If only one neighbour is active, assign the label of the neighbouring pixel;
3. If more than one neighbours are active, assign one of the labels and record label equivalences.

Following the complete traversal of the image, assign a single label to all members of equivalence classes. The resultant image will contain G disjoint regions within the image.

**Results**

The methodology introduced in the Material and Methods section was applied to the two publicly available FCM datasets discussed earlier. The gating solutions produced from the proposed methodology are compared to the model based clustering with t-distributions methodology of Lo et al. (14). The approach by Lo et al. (14) has previously been compared to manual gating by FCM experts, performing in line with manual gating solutions and has also been shown to be less restrictive than k-mean clustering solutions.

***Application to Rituximab data***

The Rituximab data has been analysed following the standard FCM data analysis practice described earlier. As mentioned earlier the data consisted of 1545 observations across four FCM variables, however 36 observations which recorded maximum intensities in either FSC or SSC were removed prior to analysis, similar to the approach by Lo et al. (14). Figure 1 (a) displays the initial clustering solution generated from a t mixture model with Box-Cox transformation restricted to selecting only one cluster. This initial gating was replicated using the MRF approach outlined in the Materials and Methods section of this paper, the resulting solution is shown in Figure 1 (b).

***Application to Graft-versus-host-disease data***

**Discussion**

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