**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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**Abstract**

Flow Cytometry technology has become an emerging state-of-the-art device in microbiology and dairy science, and is also used extensively in medical diagnostics. Unfortunately, the lack of a robust statistical analysis toolbox for flow cytometry has restricted the deployment of this world-leading sensor technology. The methodology outlined in this paper utilises the Ising model as an image segmentation tool, allowing for a distribution free method for the identification of homogenous cell populations in cytometry data. The implementation of the methodology is discussed whereby simulated annealing and hierarchical approaches are applied to improve computational efficiency. Finally, the use of two publicly available datasets allows the methodology to be evaluated by experts and in comparison with other automated gating solutions in the literature.

**Introduction**

Although the instrumentation and underlying technologies supporting flow cytometry (FCM) have advanced significantly in recent years (1, 2), the development of automated data analysis tools remain elusive, the absence of which has perhaps restricted the deployment of this state-of-the-art sensor technology (3). The conventional method for analysing FCM data 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, 4-9), and would, ideally, 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 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. In order to reduce the subjectivity and hence variability associated with manual gating a reliable statistical methodology with appropriate software implementations is needed.

Gating FCM data is an application of statistical cluster analysis where the requirement is to identify cell populations that are homogenous in nature. 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) but classical k-means methods only allow cells to belong to one single cluster. To overcome this restrictive feature, a fuzzy k-means clustering approach that allows cells to belong to multiple clusters with an associated probability of cluster membership, has been proposed (19). However, the criterion employed to select the ‘best’ clustering solution in these approaches can unduly restrict the shape, size and orientation of identified sub-populations, which can result in failure to identify “true” clusters. Some 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. These approaches require the availability of training data which often renders them unsuitable in a field where training data is not always available.

Model-based clustering has gained prominence as a statistical tool for the gating of FCM data (13,14). Model-based clustering methods assume that sample observations arise from a mixture of one or more probability densities where each probability density represents a unique sub-population or cluster (24-27). Typically it has been assumed that each mixture component follows a *p*-variate Gaussian distribution, with the number of clusters identified via a suitable information criterion (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 exploited the inherent structure of the underlying data. The use of an analogue-to-digital converter (ADC) to process the individual wavelength intensities in FCM scanners means that the output data are in reality discretised values. 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). This underlying structure provides a lattice grid of unique pairs of discretised wavelength intensities in a 2D analysis, thus the 2D graphical representation can be expressed as an image, where each pixel has a Bernoulli outcome. This paper develops a methodology based on the Ising model, which has been used extensively in the field of image processing (30-34) for removal of noise/outliers and the identification of sub-images, that provides a solution for automatic gating by exploiting this 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***

For expository purposes, the proposed automated gating solution will be exhibited by using two publicly available FCM datasets: the rituximab data from (35); and the Graft-versus-Host-Disease (GvHD) data from (36). The use of these two FCM experiments will demonstrate the utility of the proposed methods and additionally permit comparison to previous published automated gating solutions (14, 37). The rituximab data consists of 1545 cells measured for FCS and SSC, along with two fluorescent markers. The GvHD data contains a sample from two individual patients each measured across four fluorescent markers with in excess of 6800 cells per sample. For complete details of the aforementioned FCM experiments see (14, 35, 36).

***Ising Model***

Markov Random Fields (MRF). The Ising Model (38) is a special case of MRF where the pixels of the underlying latticetake outcomeswhere 0 indicates no observed cell at the current pixel and 1 infers at least one cell observation for the current pixel. The Markov property infers that for a given pixel its probability of taking either outcome is dependent only on its four nearest neighbours, as shown in Figure 1. Letbe the neighbours ofand define as the number of neighbours ofwith outcome The Ising model is then defined mathematically via full probability conditionals as

whereis an interaction strength such thatencourages neighbours to adhere to the same pixel value andis the previous configuration of the lattice grid. In addition, the probability space is also discrete with the probability of an interior point taking the value ofbeing restricted to five unique values for each value of

\*\*\*\* Figure 1 near here \*\*\*\*

Model Fitting. A number of Markov Chain Monte Carlo procedures have been developed to fit MRF, the Metropolis algorithm (39) is used here as it has been shown to achieve convergence quicker than other approaches (40). The Metropolis criterion relies on the calculation of the change in energy from changing a single element in the current configuration. If the change in energy is negative the new configuration is accepted, however if the energy change is positive the new configuration can be accepted with a probability governed by the current temperature of the system (41). Simulated Annealing (SA) further improves the computational efficiency of identifying a global minimum in the global energy function defined on the lattice (42, 43). The SA approach allows for a varying temperature which governs the probability of acceptance. Initially with a high temperature practically all changes are accepted but as the method approaches an equilibrium lower temperatures allow changes only which significantly decrease the energy of the system. A hierarchical MRF approach (46-49) is used to overcome the issue of sparsity often observed in FCM data. The dimensions of the lattice grid on which the data lie are reduced, resulting in aggregated data. The Ising model with SA models this lower resolution grid which in turn provides the starting point for the resolution at the next level, the method continues until a solution at the original resolution level is obtained.

***Gating***

Following the use of the Ising model the boundary regions of clusters in the resulting probability map is addressed. Connected component labelling (50) is an established pattern recognition tool used extensively in the identification of disjointed regions in binary, and with adaptations non-binary, images (51-53). 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. Following a complete traversal of the image, 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.

Similar to the work of Lo et al. (14) and Hahne et al. (15), we focus on a sequential clustering 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 and not relevant to the analysis. However, the proposed gating strategy can be applied to any pair of recorded FCM variables. In addition, doublets are removed prior to analysis by removing all cells which record FSC or SSC values of maximum intensity.

***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 2 (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 2 (b).

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

**Discussion**

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1. Kevin C. Brosnan was supported by an Irish Research Council Government of Ireland Scholarship (GOIPG/2014/19). Kevin Hayes was supported by the Science Foundation Ireland Research Investigator's Award (SFI-12/IA/1683). [↑](#footnote-ref-1)