**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. We propose a statistical model which automates the gating of cytometry data, which is sensitive to the underlying structure of the collated data. The methodology utilises the Ising model as an image segmentation tool, allowing for a distribution free method to identify homogenous cell populations, removing restrictions of shape, size and orientation often imposed by clustering algorithms. The implementation of the methodology is discussed whereby simulated annealing and hierarchical approaches are applied to improve computational efficiency. The approach is demonstrated on two publicly available datasets, providing comparison to other automated gating procedures in the literature. This methodology moves beyond the manual gating approach often employed, allowing for reproducible results without imposing restrictions on the features of identified cell populations.

**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). The identification of cell populations in one pair of cytometry variables may not be consistent across all pairs of recorded variables. This information loss can result in conflicting cell populations identified across varying pairs of variables. In order to reduce the subjectivity and hence variability associated with manual gating a reliable statistical methodology with appropriate software implementations is required.

Gating FCM data can be viewed as an application of statistical cluster analysis where the requirement is to identify cell populations that are homogenous in nature within groups and heterogeneous between groups. A variety of statistical clustering techniques have been applied for gating FCM data (9, 15, 16). A complete review of these approaches can be found in Lo et al. (14). In recent years, model-based clustering has gained prominence as a statistical tool for the gating of FCM data (13,14), whereby it is assumed that sample observations arise from a mixture of one or more probability densities each representing a unique sub-population. The distributional properties imposed by model-based clustering are restrictive in the size, shape and orientation of identified sub-populations and often requires transformation of FCM data before being employed.

However, 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 binary outcome. While several approaches for the automation of FCM gating have been proposed, none to date have exploited the inherent structure of the underlying data. 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 utilises the underlying lattice grid structure of the observed data to infer a probabilistic field on which the data lie via the Ising model. The fitting of the Ising model can be computationally intensive and sensitive to initialisation conditions. As such simulated annealing and hierarchical Markov random fields are employed to overcome these issues. The Ising model has also been used widely in the processing of images, both for the removal of noise and the identification of objects (31-34), objectives similar to the practice of gating by cytometrists. The methodology is demonstrated on data from two publicly available cytometry experiments, followed by a discussion on the advantages and limitations of the methodology in its current form.

**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. The GvHD data used in this paper has already undergone the initial gating procedure and as such is only subject to the second gating in the results. 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 then provides a probability of a pixel being an active cell given the current configuration of the lattice grid. The model is defined mathematically 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 **(REFS)**. The Metropolis algorithm (39) is used here as it has been shown to achieve quicker convergence 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. The optimum solution is found when the energy of the system is minimised. As such, if there is a decrease in energy the new configuration is accepted, however if there is an increase in energy the new configuration can be accepted with a probability governed by the current temperature of the system (41). The temperature of the system acts as an attractive force of adjoining pixels, a positive temperature encourages adjoining cells to be in the same state while a negative temperature encourages the opposite. 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 almost all changes are accepted but as the method approaches an equilibrium lower temperatures allow only changes 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***

We have analysed the Rituximab data using the methodology outlined above, in line with the sequential clustering approach used in practice. The 1545 cells included in the data measured across four FCM variables were included in the analysis, 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 (14). 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). This initial clustering on the FSC and SSC variables provides the sub-population of interest for the second gating stage of the Rituximab data.

The second stage of the gating, the sub-population identified in stage one is gated using the two cytometry variables, namely 7 AAD and Anti-BrdU FITC. Figure 3 (a) presents the t-mixtures solution to the second gating stage fitted with three clusters as motivated by the BIC and shown in Lo et al. (14). The methodology proposed in this paper is also applied to stage two of the gating procedure. The number of clusters to identify is not required a priori utilising this methodology removing additional user subjectivity from the analysis. The resulting cell populations are shown in Figure 3 (b), while the probability map of the complete grid is shown in Figure 3 (c) showing the probability of regions of high activity and the boundaries of these regions.

***Application to GvHD data***

**Discussion**

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**Figures and Tables**

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)