FLOPTICS: A Novel Automated Gating Technique for Flow Cytometry Data

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Abstract: Flow cytometry (FCM) involves the use of optical and fluorescence measurements of the characteristics of individual biological cells, typically in blood samples. It is a widely used standard method of analysing blood samples for the purpose of identifying and quantifying the different types of cells in the sample, the result of which are used in medical diagnoses. The multidimensional dataset obtained from FCM is large and complex, so it is difficult and time-consuming to analyse manually. The main process of differentiation and therefore labelling of the populations in the data which represent types of cells is referred to as Gating: gating is the first step of FCM data analysis and highly subjective. Significant amounts of research have focussed on reducing this subjectivity, however a faster standard gating technique is still needed. Existing automated gating techniques are time-consuming or need many user-defined parameters which affect the differentiation to different clustering results. This paper presents and discusses FLOPTICS: a novel automated gating technique that is a combination of density-based and grid-based clustering algorithms. FLOPTICS has an ability to classify cells on FCM data faster and with fewer user-defined parameters than many state-of-the-art techniques, such as FlowGrid, FlowPeaks, and FLOCK.

1 INTRODUCTION

Flow cytometry (FCM) is a high-throughput technology that is used to identify characteristics of cells by using the concept of cell-scatter measurement and light emission after receiving a laser beam stimulation (Bio-Rad, 2018). The technique provides a set of chemical and physical characteristics for each individual cell in fluid samples such as blood (Lo et al., 2008) and can process large numbers, giving a detailed information on size and distribution of the different cell populations. It is a standard diagnostic tool in general healthcare and has been widely applied in medical research, especially in haematology and immunology, and broadly adopted in clinical environments to diagnose and monitor treatments, such as: leukaemia, chemical healing responsiveness, and stem cell transplantation monitoring (Jahan-Tigh *et al.*, 2012). The process provides multi-dimensional data, including relative size, relative granularity, and relative fluorescence intensity (BD-Biociences, 2002). The data is highly complicated and difficult to analyse as a result (Bashashati and Brinkman, 2009).

Flow cytometry measures individual cells by compressing them into a narrow stream of fluid passing through at least one laser beam, with detectors to measure transmission, reflection, scatter and fluorescence emission. Cell properties that can be measured include relative size, relative granularity, and relative fluorescence. This technique was developed over 40 years ago but was limited initially because the cytometer was too large, difficult to maintain and an expensive instrument. As with many modern pieces of equipment (Robinson *et al.*, 2012), FCM is now more accurate, cheaper, and more convenient to use, hence its wide application in clinical research, particularly haematology and immunology.

Flow cytometry is able to detect cells from 0.2 microns to 150 microns in diameter, but actual capability depends on the equipment used (Rowley, 2019). In FCM, the fluid containing the cells is driven through a narrow nozzle, with the resulting ejected stream or droplets thin enough to have only one cell at a time passing through the laser beams. Typically, scattered light and fluorescent emissions from each cell are measured by a detector; light scattered by less than 5 degrees is called forward scatter (FSC) and is used to identify the size of cells, while larger deflections are called side scatter (SSC) and are used

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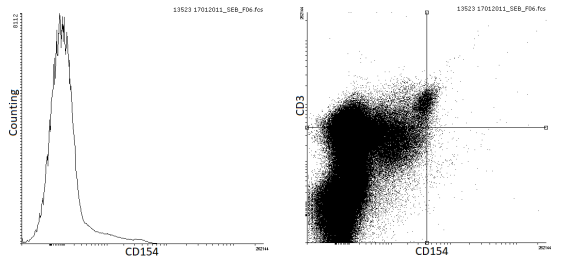
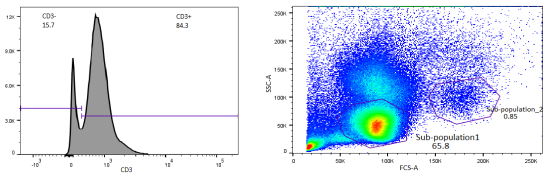


a. Histogram *b. Scatter plot*

Figure 1: Flow cytometry data examples. (a) a histogram of the number of cells measured at different fluorescent intensity values for the CD154 marker. (b) a scatter plot of the fluorescent intensity values for the CD3 versus the CD154 marker, used for identifying smaller populations, with the quadrant markers demonstrating that most cells have a low response to both CD154 and CD3.

to measure granularity and membrane roughness (World Health Organization, 2009). Different fluorescence molecules or "markers" are used to label particular types of cells to improve the identification and quantification of different populations and sub-populations. The standard identification system for markers is referred to as Cluster of Differentiation (CD).

Flow cytometry data is a multidimensional dataset and the data is generally displayed in one or two parameters (Moloney and Shreffler, 2008). For one parameter, it can be displayed as a histogram with the parameter value on the x-axis and the frequency (number) of cells on the y-axis (Figure 1a). For two parameters, the data is displayed as a scatter plot, with points representing the cell as an (x,y) pair of the values of the two parameters (Figure 1b). Up to 50 cell parameters can be determined (Lee *et al.*, 2017), with the number of features dependent on the flow cytometer and experimental design. Viewing the entire dataset is involved and complex.



a. 1D histogram *b. 2D scatter plot*

Figure 2: Manual gating examples using either drawn lines in 1 dimension (histogram) or polylines in 2 dimensions (scatter plot), to visually identify populations.

After obtaining the data, an expert operator identifies the populations - known Gating. Gating is the process of identifying cells by drawing shapes around populations (Bashashati and Brinkman, 2009), as shown in Figure 2. The expert needs to know about the characteristics of the cells of interest, and the populations and sub-populations of cells before starting the analysis.

Manual Gating is, therefore, highly subjective and time consuming - with machine learning being proposed to support this process (Lo *et al.*, 2008).

FCM data is so large and complex that it is difficult to analyse without computational tools. There are three main problems in FCM analysis; firstly, manual gating (identify cells of interest) is highly subjective (Lo *et al.*, 2008); secondly, sometimes the number of key events is very low (Groeneveld-Krentz *et al.*, 2016), which makes them harder to detect and may result in false positives; thirdly, manual gating is a time consuming process (Rahim *et al.*, 2018), especially when the number of parameters and cells are large. Although some applications have been developed to help clinical experts, flow cytometry data analysis application still have limitations, as mentioned before. The paper presents the application of machine learning techniques to implement a novel automated gating method which can provide appropriate clustering of cells in blood samples.

2 METHOD

Ye and Ho, 2018 proposed a state-of-the-art automated gating technique, FlowGrid, and claimed higher accuracy and better time efficiency compared with flowPeaks (Ge and Sealfon, 2012), FlowSOM (Van Gassen *et al.*, 2015), and FLOCK (Qian et al., 2010). However, FlowGrid still has the problem with requirement of too many user-defined parameters.

The method proposed here has the aim of improving the performance of FlowGrid, by reducing both process time and user-defined parameters. This improved method, the FLOPTICS algorithm, begins by partitioning data into equal-sized grids for each dimension ('bins') – with then only non-empty bins being processed as data points. An example of partitioning 2-dimensional data in this way is shown in Figure 3. Partitioning data is not appropriate for low density datasets, but FCM data is always high density (as can be seen in Figure 1 and 2), so the accuracy results of gating are acceptable and the run time is faster than many state-of-the-art techniques.

2.1 DBSCAN

Density-Based Spatial Clustering and Application with Noise (DBSCAN) was proposed by Ester *et al.* (1996). DBSCAN is a density-based algorithm for

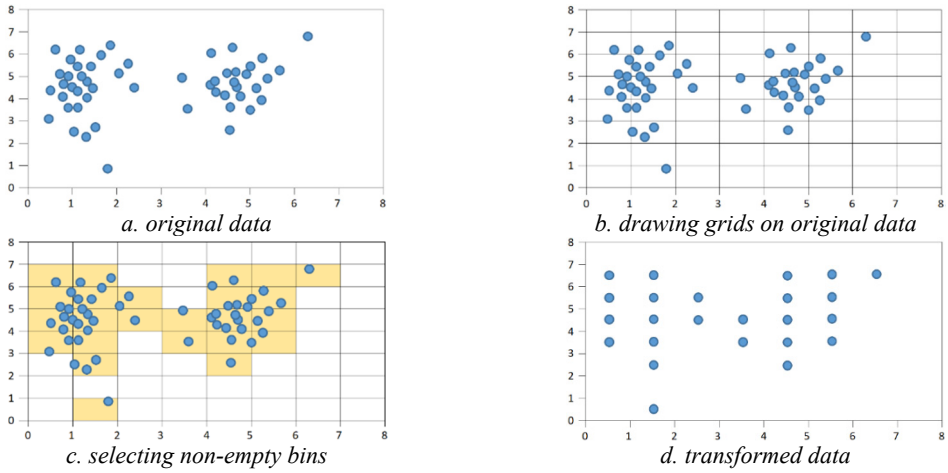


Figure 3: Partitioning 2-dimensional data into equal-sized bin for each dimensions.

clusters, so it can identify non-convex shapes. Methods based on density must have some parameters defined in advance, and for DBSCAN, there are two such parameters (defined by the user): *Eps (ε)* and *MinPts*. A point that is considered as a member of a cluster needs to have at least one neighbour (another data point) where the distance between the pair is closer than ε. In other words, the data point p is a neighbour of point q when the distance between p and q is less than or equal to ε. *MinPts* is the minimum number of neighbours for a data point to be a member of a cluster. The algorithm for DBSCAN clustering can be summarised as:

Step 1: Label all data points as core points, border points, and noise points.
Step 2: Treat a core point as the centre of a group.
Step 3: Merge each group together if they have at least one overlapping neighbour.

A *core point* is a point that has at least MinPts neighbours. A *border point* is a point that has less than MinPts neighbours, but is a neighbour of at least one core point. A *noise point* is a point that is not a neighbour of any core point.

Although DBSCAN is able to identify convex shapes, the number of clusters does not need to be defined in advance and, has the ability to identify noise - which leads to more robustness than partition-based clustering. However, it only works properly for datasets with uniform densities and parameters need to be defined before clustering is performed.

2.2 FlowGrid

This framework is a combination of DBSCAN and a grid-based clustering algorithm that provides high accuracy. DBSCAN can detect outliers and identify arbitrarily-shaped clusters. FlowGrid combined the benefits of DBSCAN and reduced computational time by using equal-sized grids, similar to the FLOCK algorithm (Qian *et al.*, 2010). Each dimension is partitioned into an equal-sized bin, so the total number of bins for d-dimensional data is $(N_{bin})^d$, where N_{bin} is the number of bins for each dimension. All data points in the same bin are treated as a single point by using a representative, which acts as an index or label for the bin; moreover, only non-empty bins are considered, which is the reason why this framework is faster than previous ones. Every Bin_i is labelled with a row of d positive numbers. For example, if $C_i = (5,2,3)$ is a coordinate of Bin_i, it means that the dataset has three dimensions, and the corresponding data points are located in the fifth bin of dimension one, the second bin of dimension two and the third bin of dimension three. Although FlowGrid is faster than many automated gating algorithms, provides high accuracy, and can deal with noise, there are still some user-defined parameters that can significantly affect the clustering result. Moreover, FlowGrid is based on DBCSCAN, meaning it is not suitable for datasets with different density distributions.

2.3 OPTICS

Ordering Points To Identify the Clustering Structure (OPTICS) was proposed by Ankerst et al. (1999). The algorithm was derived from DBSCAN in order to deal with the need for two parameters, which could provide different clustering results for different density thresholds. However, OPTICS does not produce an explicit clustering; instead, it generates an ordering density clustering. The main idea behind

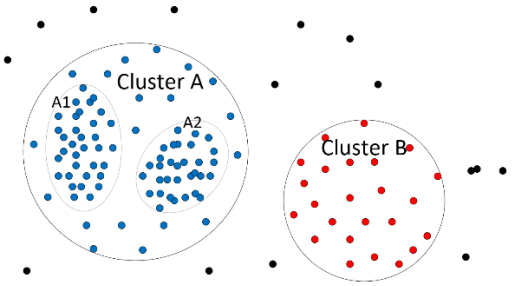


Figure 4: Higher-density clusters A1 and A2 are completely within lower-density cluster A.

these algorithms is that higher-density clusters are completely contained in a lower-density one, as shown in Figure 4. Local higher-density clusters, therefore, should be processed first. Key terms involved in OPTICS algorithm are defined as follows:

Core-distance: Assuming p is an object in the dataset, ε is a value of the distance between two objects, $N_\varepsilon(p)$ is a set of neighbours of object p, and *MinPts* the minimum number of neighbours, then $core\text{-}distance_{\varepsilon,MinPts(p)}$ is equal to:

- Infinity or undefined, if the cardinality of $N_\varepsilon(p)$ is less than *MinPts*.
- Otherwise, the minimum distance from p to its neighbour that can cover at least *MinPts* members.

Reachability-distance: If p and o are objects in the dataset, ε is the distance between two objects, $N_\varepsilon(p)$ is a set of neighbours of object p, and *MinPts* the minimum number of neighbours, then $reachability\text{-}distance_{\varepsilon,MinPts(o,p)}$ is equal to:

- Infinity or undefined, if the cardinality of $N_\varepsilon(p)$ is less than *MinPts*.
- Otherwise, *Max (core-distance(p), distance(o,p))*.

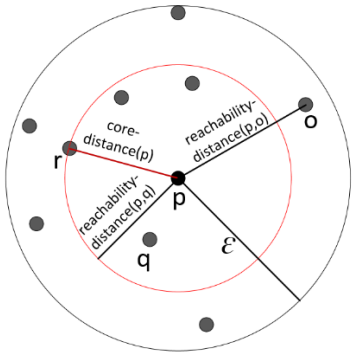


Figure 5: The difference between core-distance and reachability-distance, given ε and MinPts = 5.

Therefore, in accordance with these definitions, *reachability-distance* must be equal to or greater than *core-distance*, as shown in Figure 5. Then, *reachability-distance*(p, q) is equal to *reachability-distance*(p, r) and equal to *core-distance*(p), while, *reachability-distance*(p, o) is greater than *core-distance*(p).

The algorithm for OPTICS clustering can be summarised as follows:

Step 1: Read an unprocessed object (p) from the dataset
Step 2: If p is a core-object, update core-distance of p
  For each $q \in N_\varepsilon(p)$
    Update reachability of object q
  Update the OrderSeeds list, which contains the objects ordered by reachability-distance (from smallest-to-largest)
  Mark p as processed
Step 3: Read an unprocessed object p from the OrderSeeds list if the list is not empty; otherwise, read the next unprocessed object from the dataset
Step 4: Repeat *Step 2* - *Step 3* until the end of the dataset

Most density-based methods, such as DBSCAN and OPTICS, can detect non-convex cluster shapes, identify noise and automatically identify the number of clusters. However, the density thresholds and other parameters need to be carefully defined, because different identification of the parameters in this method could lead to different clustering results.

2.4 FLOPTICS

For the algorithm presented here, termed FLOPTICS, data is partitioned into equal-sized bins, and the data is clustered using the OPTICS algorithm (Ankerst et al., 1999). The radius distance (ε) has to be defined by the user, as in the DBSCAN (Ester et al., 1996) algorithm, but OPTICS can provide the optimal value of ε by showing the structure of data. Therefore, the number of user-defined parameters for FLOPTICS is fewer than FlowGrid, which is based on DBSCAN. The FLOPTICS algorithm can be summarized as follow:

Step1: All data points are partitioned into equal sized bins for each dimension
Step2: Only non-empty bins are processed
Step3: Read an unprocessed bin (b) from the dataset obtained from Step 2
Step4: If b is a core-bin, update core-distance of b
  For each $a \in N\varepsilon(b)$,
    update reachability of object a
  Update the OrderSeeds list, which contains the objects ordered by reachability-distance (from smallest-to-largest)
  Mark b as processed

Step5: Read an unprocessed bin *b* from the OrderSeeds list if the list is not empty; otherwise, read the next unprocessed bin from the dataset
Step6: Repeat Step 3 - Step 5 until the end of the dataset

The key terms involved in the algorithm are defined:

- $N_\varepsilon(b)$ is a set of neighbours of bin *b* for radius distance value ε, identified by the user
- *Core-bin* is the bin that its number of neighbour (regarding the radius ε) more than or equals to *MinPts*, which is identified by a user
- *Core-distance(b)* is the minimum distance that lead the number of neighbour of bin *b* reach *MinPts*
- *Directly connected: Bin a* is directly connected to *Bin b* if $Distance(a,b) \leq \varepsilon$
- *Reachability-distance:* If *b* and *o* are bins in the grid space, ε is the distance between two bins, *Nε(b)* is a set of neighbours of bin *b*, and *MinPts* is the minimum number of neighbours, then *reachability-distanceε,MinPts(o,b)* is equal to:
  - Infinity or undefined, if the number of members in *Nε(p)* is less than *MinPts*
  - Otherwise *the maximum of core-distance(b)* or *distance(o, b)*
- The *OrderSeeds list* is the list (queue) of bins in the grid space ordered by reachability-distance

3 RESULTS AND DISCUSSION

DBSCAN, OPTICS, FlowGrid and FLOPTICS were applied to a synthetic dataset, is generated to mimic a real FCM dataset with control over data features. The experiments were conducted on a computer with specification as follows: Intel(R) Core(TM) i7-6700 CPU @ 3.40GHz; RAM 16.0 GB; Operating System - Windows 10 Enterprise, 64-bit.

3.1 Reference Dataset

Patterns or clusters in real sample datasets obtained from different donors will be similar but different, even they are obtained from the same flow cytometry experimental setup. The cell populations in any sample generally have a normal distribution for the measurements of any given marker or optical characteristic. Therefore, cluster shapes formed from two normally distributed value sets are usually found but can be symmetric or asymmetric depending on the donors and markers used. The clusters might be, for example, circle-shaped with different radiuses, or cigar-shaped with different widths, heights and angles and can be different from donor to donor. In order to provide some clear comparative analysis as well as to explore the limitations of the methods, imitative datasets were generated based on model blood sample, rather than randomly choosing a donor blood sample. The parameters of the data could then easily be modified to test the performance of each method.

3.2 Generation of Imitative Datasets

The imitative datasets used in the experiment were 2-dimensional datasets generated by the function *rmvnorm (n, mean, sigma)* in RStudio 3.5.2; this function randomly generates data from a multivariate normal distribution, which is often found in FCM data. For this function, three arguments are required: the number of data points (*n*), an average of the data (*mean*), and a covariance matrix (*sigma*). The structure of the imitative datasets consisted of three clusters for each dataset. The number of data points in Clusters 1, 2 and 3 were 5000, 2500 and 2500 respectively. They were generated with four different argument sets, which mean four different overlapping levels (shown in Table 1) and generated three times for each set of arguments; in total, 12 datasets were used in experiments. Examples of these imitative datasets are shown in Figure 6.

Table 1: Parameter values for the generation of the imitative datasets used in this work.

Cluster	Sigma (Covariance matrix)	N	Means (Centres)							
			Level 1		Level 2		Level 3		Level 4	
			x	y	x	y	x	y	x	y
1	[(6,15), (15,120)]	5000	5	35	3	30	1	25	-1	20
2	[(2,0.3), (0.3,5)]	2500	-5	-10	-5	-10	-5	-10	-5	-10
3	[(3,2), (2,10)]	2500	16	1	12	1	8	1	4	1

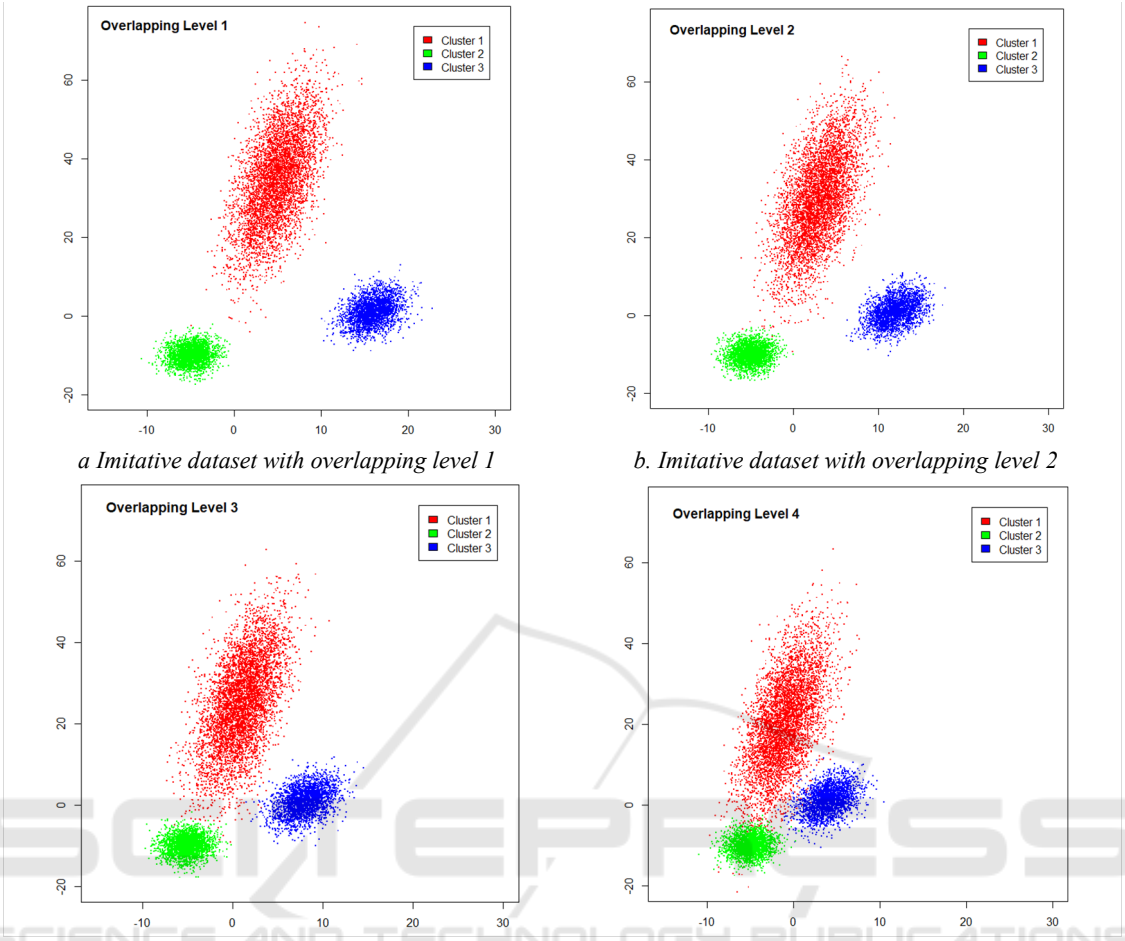
3.3 Results

The datasets were clustered using DBSCAN, OPTICS, FlowGrid, and FLOPTICS, with user-defined parameters shown in Table 2. The values of ε for DBSCAN and FlowGrid which provided the best average accuracy results were selected (0.8 and 6.0 respectively).

Table 2: The parameter values for each technique.

DBSCAN	OPTICS	FlowGrid	FLOPTICS
$\varepsilon = 0.8$ *MinPts* = 10	ε = optimal *MinPts* = 10	$\varepsilon = 6$ *MinDenB* = 3 *MinDenC* = 40 *Bin_size* = 100	ε = optimal *MinPts* = 10 *Bin_size* = 100

All techniques were implemented and run on RStudio 3.5.2, and the result are presented in Table 3.



a Imitative dataset with overlapping level 1

b. Imitative dataset with overlapping level 2

c. Imitative dataset with overlapping level 3

d. Imitative dataset with overlapping level 4

Figure 6: Scatter plot of imitative datasets.

Table 3: The results of applying the analysis methods techniques to the datasets.

Techniques	**Average accuracy** (%)				**Overall average accuracy** (%)	**Average Runtime** (milli-second)
	Overlapping dataset Level					
	1	2	3	4		
DBSCAN	94.99	94.70	94.80	62.70	86.80	6,728.60
OPTICS	99.93	99.75	98.60	92.07	97.59	1,961.12
FlowGrid	96.00	95.75	95.01	93.59	95.09	723.80
FLOPTICS	99.87	99.49	97.60	90.45	96.85	265.88

4 CONCLUSIONS

According to the results, OPTICS provided the best average accuracy of 97.59%, though FLOPTICS gave a higher accuracy result than DBSCAN and FlowGrid. Although OPTICS gave the highest accuracy, it was approximately 7.4 times slower than the FLOPTICS technique. FLOPTICS was the fastest technique applied to the imitative datasets, compared with DBSCAN, OPTICS, and FlowGrid. In terms of the number of user-defined parameters, FLOPTICS requires two parameters, which are *MinPts* and *bin_size*, while FlowGrid requires four parameters, which are ε, *bin_size*, *MinDenB*, and *MinDenC*. In conclusion, FLOPTICS has better performance than comparative state-of-the-art automated gating techniques.

5 FUTURE WORK

Although the FLOPTICS algorithm provides better accuracy and a fast run time, its performance can be further improved. In the process of partitioning data into equal-sized bins, only non-empty bins are processed, but both high-density bins and low-density bins are treated equally; moreover, core points are

identified by consideration of the number of neighbours. An improvement would be to identify core points not only by the number of neighbours, but also the density of individual bins. Moreover, the proposed technique is tested on a single specialised machine. The next stage will be to revise the algorithm to be machine-independent.

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