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MMA Technology

Robert Tornai

Department of Computer Graphics, University of Debrecen
e-mail: rtornai@inf.unideb.hu

Abstract

MMA stands for the Multiple Microbead Assay term. The basic problem is to evaluate the results of flow-cytometry measurements. Our team has developed two softwares in corporation with Soft Flow Kft. The softwares support the field of microbead technology inside the flow-cytometry and can be used to evaluate measurements related mainly to immunology. Flow-cytometry is a very young technology, it was invented hardly a decade ago. In fact it is a diagnostic and analytical method.

This paper gives the problem's description and skeleton of the solution how to handle Multiple Microbead Assay measurements. It will give an overview of the most important equipment producer corporations, the main steps of a measurement, the structure of a List Mode (LM) file, the architecture of the developed programs and the current state of the project.

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Key Words and Phrases: flow-cytometry, experiment planning, microbead assay measurements

1. Introduction

The basic problem is to evaluate the results of flow-cytometry measures. The softwares were developed in corporation with Soft Flow Kft. The softwares support the field of microbead technology inside the flow-cytometry and can be used to evaluate measurements related mainly to immunology. Flow-cytometry is a very young technology, it was invented hardly a decade ago. In fact, it is a diagnostic and analytical method.

2. Flow-cytometers

To perform a flow-cytometry measurement, a flow-cytometer machine is needed. This machine can be a general-purpose (used for research), an independent or a special hardware, which is the main part of a haematologic machine.

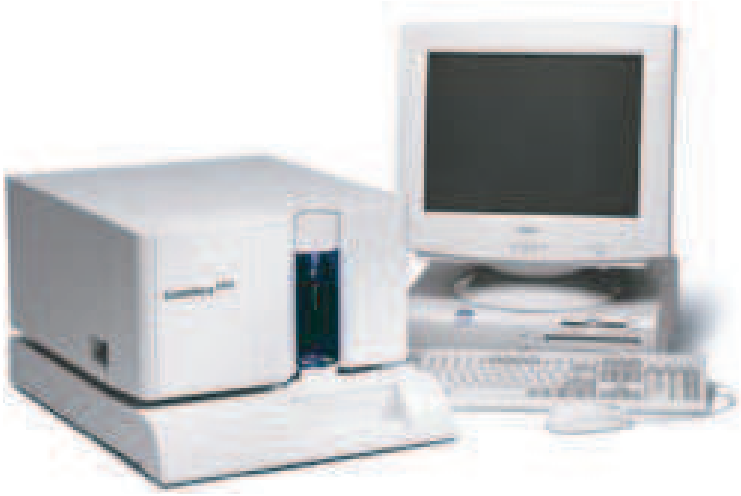

Figure 1: Picture of a Luminex 100 system.

The most well known flow-cytometer manufacturers are: Beckton Dickinson, Luminex, Coulter and Bio-Rad.

3. Steps of a measurement

The guiding principle is as follows:

- First of all, the assay to be examined, which can be a group of cells or a microbead mixture, mixed with the appropriate reagents.
- After this step, the obtained mixture is mixed with an organic fluorescent stain that is connecting to the reagent.
- The mixture inserted into the machine.
- The diluted mixture goes into the exposition box through a capillary.
- The high speed mixture is lit by laser in this box. The laser light is reflecting in different directions or absorbing in it. In the second case the laser excites the stain moleculas, so they begin to emit light.
- The "event" phrase will be used for the assay that has just passed by the laser beam.
- The intensity of the reflected light is percepted by some well placed sensors.

- The signs are forwarded to the cytometer's processing unit. The signs are digitized here. Older machines use 8, 10, 15, 16 bits, the newest models are capable to use 32 bits. Usually a logarithmic conversion is possible to achieve the best results.

- The digitized channel values get to the collector machine through the data cable of the cytometer. This is usually a PC, where a special collector software interprets the data. A file is created for each measurement. This file is called List Mode file (abbreviated form: LM file).

4. List Mode files

List Mode files usually follow the Flow Cytometry Standard (abbreviated form: FCS). The FCS structure contains:

- Header (sequence of ASCII characters): contains ID-value pairs separated by one or two special characters (called separators). Separators are producer defined. Some of them follow the standards, others are uniquely defined.

- Constant values: eg. powering factor, digital conversion resolution (bit depth), name of the channels, date and time of the measurement, etc.

- Number of events: number of the percepted moleculas.

The following parts are bytes making up data sequences that can be interpretered by the previous informations. These data sequences store the converted, digitized channel values for the events in time sequence. Nowadays CSV-based (Comma Separated Values) List Mode files are getting widespread among Luminex instruments. They include the intensity values assigned to the events one after other. The new version of the software is capable to recognize and process these files, too.

The software does not support haematologic machines, since these machines were developed for special haematology tasks and their results can be interpretered directly without using external software or hardware. The software supports flow-cytometers which are mainly used by researchers for different type of measurements. Hospitals do use these kind of machines as well, however the current software version do not have licence nor have certificate to use it in such environments. The reason is that hospital usage was not a purpose. The most important consideration was to fulfill the demands of the research usage and the final product perfectly fit these demands.

5. MMA technology

The advantage of the MMA techology is that several reactives can be used in a measurement, provided that several stains are used with homogeneously mixed

beads that are covered with different reactives. This way the amount of the necessary assay (blood, plasma or tincture) will be significally smaller. The beads are identified by the emission of the contained fluorescent stain. If only one stain is used with more different concentrations, then maximum 10-16 beads can be distinguished (with little overlay) from the data of one channel. Two channels' data is needed when two stains are used, so the maximum number of distinguishable beads rise to 256. Theoretically more stains can be used as well, but there is a biological limit to create a so complicated system. (Even the usage of three stains is not common.) The number of distinguishable beads can be extended by using different sized beads, namely the size of different beads corellate with side scatter of the laser beam. The software firstly recognize the same sized beads, which is done with the help of the side scatter channel (SSc). The second step is to distinguish the events by analytes using an one or two dimensional area division method. The segregated patches are called clusters.

6. AS files

The ASF Creator software yields an AS file that supports a kit. It contains:

- Type of the evaluation (qualitative, quantitative, IS)
- A name that describes the function
- Producer defined catalog ID
- Series identifier (Lot number)
- Unique identifier number
- Supported cytometer's name
- Character series that identifies the cytometer in a List Mode file's header
- Stain (clustering) identifer parameters
- Size (SSc) identifier parameter
- Analytical (reporter) parameter
- Reactive (analyte) list in the order defined by the clustering procedure
- Bead list in the order defined by the clustering procedure
- Concentrations of the control samples by analytes or the COI of them and the tolerance
- Conditional messages' types, bounds and the texts of the messages

In quantitative case:

- The type of the function to be fitted
- Concentrations of the standard samples by analytes and the unit of the concentration

In qualitative case:

- Cut-off values of the positive and negative control samples by analytes

In Instrument Standard case:

- Lack of the clustering parameters, stain list, control samples and conditional messages
- Relative Fluorescent Intensity (RFI) value for each bead

7. The SAT Software

Steps of an experiment:

- The researcher decides the type of the experiment. He gives the reactives and decides whether qualitative or quantitative measurement is needed. Instrument standards can be used to verify the integrity of the cytometer.
- The researcher selects the kit that satisfies his needs and places the order. He has to import the AS file of the kit into the SAT program. Now the kit is ready for use in experiments.
- Assays of a kit can be used in different ways.
  - Batch assay: A given test has to be applied for samples obtained from many different sources. The end user can place test samples in a Batch assay.
  - Random assay: Used for patients. Assays for the different tests have to be assigned to the appropriate patients. Then, many different tests can be applied to a given sample obtained from one source.
- A researcher can add test samples to an assay. The amount of the other samples is defined in the AS file. These samples that can not be modified are:
  - Standards - base points of the curve used for convert the concentration of intensity at quantitative evaluation. An intensity value associated with this sample in the LM file having known concentration gives the second coordinate of the base point.
  - Quantitative Control - used for verification, shall be in a range, else the evaluation process gives an error.

  - Qualitative Control - verifier samples of the qualitative evaluation, they can yield positive or negative results. They have to be in a range of known intensity values, else they lead to errors during the evaluation.
  - Instrument Standard - unstained calibrating beads. The usage of the instrument standards can be turned off.

- The samples can be arranged on a Microbead Array Plate, or on a Rack. A plate has 96 or 384 wells in a matrix arrangement. Rows are identified by letters, and columns are identified by numbers. The software has several possibilities to automatically arrange the samples. For better results, replicates can be asked for any sample, where the replicate is from the same tincture. Each replicate has an own well in the plate. Then, a map can be printed for the technician. This map shows which tincture must inserted in which well.

- Now comes the measurement. The software does not support it directly, the LM files shall be created with the cytometer's own, special data storing system.

- LM files shall be copied into one folder and the Data File Assignment option can be used for associating assay replicates with List Mode files. If the LM files have ordered names, then automatic assignment can be used. This assignment can be modified manually if necessary.

- The report's form and appearance farthest at this point must be decided. At this point can be fixed the object of interest and the level of detail. Besides the arrange of the measure results can be set. Reports that are generated with the software are tipically 5 to 60 pages long. Two kind of report can be generated, a shorter "Summary Report" and a detailed "Full Report". The same options are used for both kind of reports.

- After everything is set up, the evaluating procedure can be started, so the List Mode files get processed. Clustering is done due to the device parameters fixed in the AS file, this means the selection of the events of one reactive. Intensity values of clusters are computed. The qualitative and quantitative methods use different evaluating procedure. In quantitative case, there is a curve fitting for the standards with the curve fixed in the AS file. With the help of the obtained curve parameters the software defines the concentration values of all other samples and classifies the control samples. In qualitative case, a cut-off index is computed from the positive and negative control samples' intensity. The test samples' intensity values get divided with this cut-off index. One control sample is mandatory, and the missing cut-off indices will be zero values.

- Possible errors during the evaluation process:

  - The instrument cannot be identified with the help of the given character string at the pattern matching phase.

- There is different number of clusters than should be.
- There is no proper curve to fit with the given function for the intensity values of the clusters at quantitative standards.

If the assays are free of these mathematical errors then the experiment can be evaluated biologically. The results are represented in a tabular form. The most important results are the derivated concentrations, the derivated cut-off indices and the deviation from the expected values at the control samples. With the help of the reporting messages the results of the test samples can easily be interpretered. All these tools together yields that a biologist researcher can plan and evaluate his experiment quickly and easily in a comfortable environment and gets the results in a form that can be read and interpretered easily.

8. Results

Researchers can prepare their experiments and produce kits for them in advance with the help of the ASF Creator and SAT softwares. Such a kit is a set of reagents and tinctures for the given experiment's type. The resulted description file of a kit is called Assay Specification file (abbreviated form: AS file).

The first conception was to have two programs. One for creating AS files and one for planning and evaluating experiments. This means two distict groups of users. Only the producer of a kit is authorized to make changes to the AS files. This is why ASF Creator is protected by an encryption algorithm. Later, as the expectations have changed, Soft Flow Kft. has decided to unite these programs together. The resulted programs make it easier to plan and evaluate an experiment for biologist researchers.

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Postal address

Robert Tornai
Department of Computer Graphics
University of Debrecen
4010, Debrecen, Egyetem tér 1.
Hungary