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MuPET-Flow: Multiple Ploidy Estimation Tool from Flow cytometry data

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► **To cite this version:**

C. Gómez-Muñoz, G. Fischer. MuPET-Flow: Multiple Ploidy Estimation Tool from Flow cytometry data. 2024. hal-04797474

HAL Id: hal-04797474

<https://hal.science/hal-04797474v1>

Preprint submitted on 22 Nov 2024

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1 **MuPET-Flow: Multiple Ploidy Estimation Tool from Flow**

2 **cytometry data**

3

4 **Keywords:** Cytometry, ploidy, genome, software, graphical user interface.

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13

ABSTRACT

14 **Summary:**

15 Ploidy, representing the number of homologous chromosome sets, can be estimated from
16 flow cytometry data acquired on cells stained with a fluorescent DNA dye. This estimation
17 relies on a combination of tools that often require scripting, individual sample curation, and
18 additional analyses. To automate the ploidy estimation for multiple flow cytometry files,
19 we developed MuPET-Flow—a Shiny graphical user interface tool. MuPET-Flow allows
20 users to visualize cell fluorescence histograms, detect the peaks corresponding to the
21 different cell cycle phases, perform a linear regression using standards, make ploidy or
22 genome size predictions, and export results as figures and table files. The tool was
23 benchmarked with known ploidy datasets, yielding consistent ploidy results. MuPET-Flow
24 produces ploidy results comparable to those from other available tools; however, MuPET-
25 Flow stands out for its user-friendly interface, which allows the whole process to be carried
26 out in one place, considerably speeding up analysis, particularly in projects involving large
27 numbers of samples.

28 **Availability and implementation:** <https://github.com/CintiaG/MuPET-Flow>.

29

INTRODUCTION

30 Ploidy (n) is the number of homologous chromosome sets in a cell. Cells can be haploid if
31 they have one set ($1n$), diploid if they have two ($2n$), or polyploid if they have three or
32 more ($\geq 3n$). Polyploidy exists in several branches of the tree of life (Otto and Whitton,
33 2000), notably in the *Saccharomyces* genus (Peter *et al.*, 2018; Gómez-Muñoz *et al.*, 2021),
34 and in plants (Čertner *et al.*, 2022).

35 Flow cytometry (FC) allows measuring the fluorescence of single cells stained with a
36 DNA intercalant, such as propidium iodide (PI). When this fluorescence is plotted against
37 the cell count (histogram of fluorescence intensity), usually two peaks corresponding to the
38 different cell cycle stages, G₀/G₁ and G₂, are recognizable. The peaks' fluorescence
39 intensity is proportional to the DNA content, which allows inferring both ploidy and
40 genome size. To achieve this, the peaks' fluorescence of query samples needs to be
41 identified and then compared to that of known standards. Such standards can be run
42 separately (external standardization) or within the sample (internal standardization) (Todd
43 *et al.*, 2018; Sliwinska *et al.*, 2022).

44 Several software tools for FC data analysis exist. Proprietary software, such as FlowJo
45 (Becton Dickinson & Company, USA), are usually tied to the equipment or require
46 payment. Open-source software, developed in R and Python programming languages, are
47 freely available and have the potential to automate the production of publication-ready
48 images but typically lack a graphical user interface (GUI) and involve scripting, which
49 might be daunting for some users. For the specific case of peaks detection and ploidy
50 estimation, two open-source tools deserve special mention as they offer a GUI. On one

51 hand, Cytotflow (Teague, 2022), implemented in Python, is a versatile FC data analysis
52 package. In this software, data can be loaded and visualized through a GUI. However, it
53 requires manual configuration, and the formal analysis must be conducted outside the
54 software (Table S1). On the other hand, flowPloidy, implemented in R (Smith *et al.*, 2018),
55 is a tool that provides powerful peaks and debris fitting, as well as gating, facilitated by the
56 inclusion of an R Shiny GUI (Chang *et al.*, 2023). Nevertheless, it still involves scripting,
57 and individual sample curation (Table S1).

58 Given the importance of FC in ploidy estimation and the limitations of the existing
59 tools, we developed MuPET-Flow (Multiple Ploidy Estimation Tool from Flow cytometry
60 data). MuPET-Flow is a GUI tool that automates file uploading and configuration, peak
61 fluorescence intensity detection, multiple histogram visualizations, peak error curation,
62 ploidy and genome size calculations, and easy results export (Table S1).

63 We benchmarked our tool using *Saccharomyces* datasets and demonstrated its
64 applicability in plant species for ploidy and genome size estimation. Additionally, we
65 compared MuPET-Flow to Cytotflow and flowPloidy. MuPET-Flow shares several features
66 and advantages with these existing tools (Table S1), but its novelty consists in automating
67 and combining features that usually exist separately, as discussed below. Notably, MuPET-
68 Flow is the only tool that allows full and simple in-app calculation of ploidy. MuPET-FC is
69 available at <https://github.com/CintiaG/MuPET-FC>.

70

MATERIALS AND METHODS

71 **Flow cytometry data acquisition**

72 *S. cerevisiae* strains included two natural polyploids CLQCA_17-111 (abbreviated as CRE;
73 3n) and CH10 (abbreviated as AVQ; 5n), used as test, and four laboratory strains, BY4742
74 (1n), BY4743 (2n), YPS128_3n (3n), and YPS128_4n (4n), serving as standards. Three
75 biological replicates of yeast cells were cultured in YPD media (Sigma-Aldrich, USA),
76 fixed in ethanol 70% and washed twice with Phosphate-buffered saline solution 1X.
77 Subsequently, the cells were treated with RNase A (EUROMEDEX, France) at 1 mg/mL
78 at 37°C for 2h, and stained with PI (Thermo Fisher Scientific, USA) at 50 µg/mL.
79 Fluorescence was measured in the MACSQuant VYB system (Miltenyi Biotec, Germany),
80 in channel FL4-A (605-625 nm). Files were gated by FSC-A vs SSC-A and FSC-A vs FSC-
81 H using CytoExploreR (Hammill, 2021). The FC files generated are available on MuPET-
82 Flow's GitHub. *Saccharomyces pastorianus* files were obtained from Gómez-Muñoz *et al.*
83 (2021), while *Solanum pseudocapsicum* files (Čertner *et al.*, 2022) were downloaded from
84 the FlowRepository (ID FR-FCM-Z45W; Spidlen *et al.*, 2012).

85 **Tool description and performance evaluation**

86 MuPET-Flow is implemented in the R package Shiny (Chang *et al.*, 2023) and comprises
87 three tabs (Fig. 1). On the first tab, 'Peaks', multiple local Flow Cytometry Standard (FCS)
88 files can be uploaded through the GUI using internally flowCore (Hahne *et al.*, 2009).
89 MuPET-Flow automatically scans the files for available channels and displays them in and
90 dropdown menu, from which one must be selected. The tool then estimates the number of
91 breaks (minimum 256) and automatically generates a histogram in the chosen channel for

92 all samples. A ‘Local Polynomial Regression Fitting’ is applied for histograms smoothing
93 (default 0.5). Peaks are detected using a local maximum algorithm by employing a sliding
94 window of a given width (default 50). Optionally, individual files can be selected through a
95 dropdown menu to display the sample's histogram and peak detection results, and the
96 smoothing level and window width adjusted by a numeric input menu. The minimum cell
97 count to call peak can be selected (default 5). The algorithm assumes that only the G0/G1
98 and G2 populations exist, and only two peaks can be used for the subsequent steps.
99 However, additional detected peaks can be explored and selected through a checkbox.

100 In the second tab, ‘Regression’, MuPET-Flow estimates ploidy or genome size using a
101 linear regression based on external standards. Therefore, the type of analysis, the number of
102 standards files, and their corresponding values should be specified. A minimum of two
103 distinct standards is necessary. The two peaks of the standards are considered for linear
104 regression, and the ploidy or genome size of both peaks of the test samples is predicted.
105 The linear regression results are displayed in a text box.

106 Finally, all generated histograms can be previewed in the third tab called ‘Summary’. In
107 case not all of the samples were individually inspected and an error is found, it is possible
108 to return to the first tab to review any parameters and selected peaks, provided that
109 regression is redone as well. All used parameters, such as ‘smoothing’ and ‘window’, as
110 well as the mean ploidy or genome size (presented as decimal and rounded values), are
111 reported. The histograms can be saved as a figure (PNG or TIFF), and the table as a
112 comma-separated value file (CSV).

113 The peaks' fluorescence of the *S. cerevisiae* dataset was also determined using Cytosflow
114 (Teague, 2022) and flowPloidy (Smith *et al.*, 2018), following their instructions. The

115 ploidies were obtained by applying the same principle as MuPET-Flow (linear regression).
116 These results were compared with a paired Wilcoxon signed rank exact test. To assess the
117 tool's execution time, we generated a mock dataset by duplicating the files of the six *S.*
118 *cerevisiae* analyzed strains for all replicates resulting in 36 FCS files (considered a
119 manageable number for visualization) with a total size of 119 MB. Using the R
120 'system.time' function, the user, system, and elapsed mean times of three runs were
121 obtained for the main application processes. Full computer specifications for developing
122 and testing MuPET-Flow are in Table S2.

123 **RESULTS AND DISCUSSION**

124 **Benchmarking with newly generated *S. cerevisiae* FC data**

125 To test our application, we determined the ploidy of two *S. cerevisiae* polyploids, CRE and
126 AVQ, from newly obtained PI-stained cells FC data. The histograms of the two tested
127 strains and four standards were visualized simultaneously (Fig. S1), and the smoothing and
128 window were adjusted for a few samples (Table S3). The estimated peaks' intensities and
129 the inferred ploidy by linear regression are reported in Table S3. The fluorescence intensity
130 varied among the biological replicates; however, there was a good agreement in the mean
131 ploidy for CRE and AVQ in the triplicates. Similarly, the rounded ploidy of the tested
132 strains was identical to that reported by Peter *et al.* (2018). Additionally, we examined the
133 execution time over the *S. cerevisiae* mock dataset consisting of 36 files with MuPET-Flow.
134 The total user time for MuPET-Flow's main processes was less than 2 seconds (Table S2).

135 **App testing with published data in other species**

136 To further assess the app's performance, we challenged it with FC data originating from
137 different instruments, fluorophore technology, and species. We utilized two previously
138 published cytometry datasets, one from the yeast *S. pastorianus* (Gómez-Muñoz *et al.*,
139 2021) for genome size estimation and another from the plant *S. pseudocapsicum* (Čertner *et*
140 *al.*, 2022) for ploidy determination. In the *S. pastorianus* dataset, we obtained a genome
141 size of the query strain *S. pastorianus* 790 of 58.64 Mb, close to the 60.1 Mb previously
142 estimated (Table S4; Gómez-Muñoz *et al.*, 2021). For *S. pseudocapsicum*, multiple peaks
143 were detected in the fruit skin file. The ploidy of the highest intensity peaks was
144 investigated, revealing correspondence with the 8n peak previously reported for this tissue
145 (Table S5; Čertner *et al.*, 2022). Thus, MuPET-Flow is primarily developed to estimate
146 ploidy in yeast; however, it can also be applied to measure genome size and in other species.

147 **MuPET-Flow comparison with open-source tools for ploidy estimation**

148 To emphasize the advantages of MuPET-Flow's pipeline, we compared it with the pipelines
149 of Cytoflow and flowPloidy, focusing on their ability to calculate ploidy over the *S.*
150 *cerevisiae* dataset. We examined whether the peaks' fluorescences and ploidies obtained
151 with MuPET-Flow differed from those obtained with Cytoflow and flowPloidy. We
152 observed a significant difference ($p < 0.05$) in the peaks' fluorescence between MuPET-
153 Flow and flowPloidy, as well as between Cytoflow and flowPloidy, but not between
154 MuPET-Flow and Cytoflow. However, no differences were observed in the inferred ploidy
155 values across the three tools (Table S6).

156 Compared to MuPET-Flow, Cytoflow (Teague, 2022) requires extensive manual
157 configuration and using the 1D mixture model for detection of the different peaks'
158 fluorescence. However, the number of peaks for the model needs to be specified, and the
159 same number is applied to all samples. Additionally, for ploidy estimation, the data needs
160 to be exported and analyzed outside the software (Table S1). Likewise, FlowPloidy
161 explicitly necessitates detecting channels, specifying the number of breaks for histograms,
162 and saving the images via scripting. Furthermore, failed models needed to be inspected and
163 corrected individually. Finally, flowPloidy is principally intended for calculating plant
164 genome size with internal standards. When external standards are used, the peaks' means
165 need to be exported and the ploidy calculated through additional operations (Table S1).

166 On the contrary, MuPET-Flow is an easy-to-use tool that assembles and automates all
167 necessary actions for ploidy estimation (Table S1). For instance, MuPET-Flow allows the
168 visualization of samples both individually and simultaneously, as in Cytoflow, and the
169 capability to correct histograms, somewhat similar to flowPloidy. These characteristics
170 combined facilitates the identification and curation of the few problematic samples, not
171 feasible in Cytoflow, and more efficient than flowPloidy which necessitates inspecting
172 every one of them. Furthermore, MuPET-Flow obtained histograms and peaks' intensities
173 can be saved as a figure and a table, always with the aid of a GUI (Table S1). An additional
174 feature of MuPET-Flow is the ability to detect multiple peaks due to its local maxima
175 algorithm, without prior knowledge of the number of peaks. The multiple peaks present in
176 certain samples can be explored and selected, as observed in the case of endopolyploidy
177 reported in *S. pseudocapsicum* (Čertner *et al.*, 2022).

178 Cytoflow, flowPloidy, and MuPET-Flow individually present several advantages and
179 disadvantages (Table S1). Nevertheless, MuPET-Flow is an attractive option as it
180 automates and facilitates many steps in ploidy estimation. It is the only tool capable of
181 performing this process within the app in a straightforward manner, while reducing the
182 need for manual curation. Overall, MuPET-Flow minimizes the time required for ploidy
183 analysis, which is particularly beneficial for projects dealing with a large number of
184 samples routinely.

185 **WEB RESOURCES**

186 MuPET-FC code and execution instructions are available at
187 <https://github.com/CintiaG/MuPET-FC>.

188 **DATA AVAILABILITY STATEMENT**

189 *S. cerevisiae* dataset is available at [https://github.com/CintiaG/MuPET-](https://github.com/CintiaG/MuPET-Flow/tree/master/example_data)
190 [Flow/tree/master/example_data](https://github.com/CintiaG/MuPET-Flow/tree/master/example_data). The *S. pseudocapsicum* dataset is available
191 <http://flowrepository.org/id/FR-FCM-Z45W>. *S. pastorianus* dataset was obtained directly
192 from authors Gómez-Muñoz *et al.* (2021).

193 **ACKNOWLEDGMENTS**

194 Flow cytometry data acquisition was performed at the IBPS Imaging Facility. The IBPS
195 Imaging facility is supported by Region-Île-de-France, Sorbonne Université and CNRS.

196 **CONFLICT OF INTEREST**

197 The authors declare no conflict of interest.

198

FUNDER INFORMATION

199 This work was supported by the Agence Nationale de la Recherche [ANR-20-CE12-0020].

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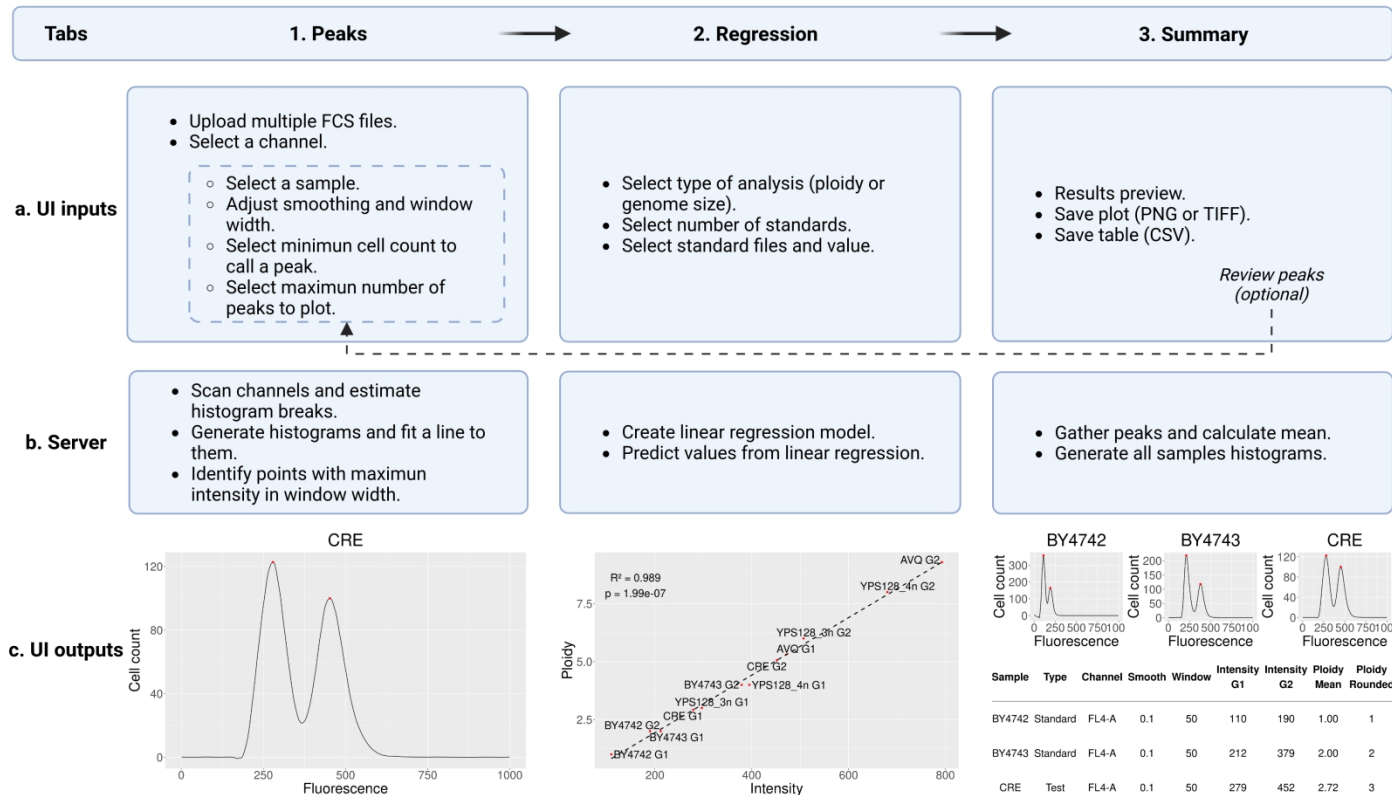
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FIGURES AND LEGENDS



243 Fig. 1. MuPET-Flow: A Shiny app for estimating ploidy in multiple samples. The MuPET-Flow workflow is divided into three tabs:
 244 'Peaks', 'Regression', and 'Summary'. The main functionalities consist of User Interface (UI) inputs, processes executed in the Server,
 245 and UI outputs. Adjusting the inputs in the dashed box is optional. Created with BioRender.com.