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# 1 **Optimization of flow cytometry parameters for high-throughput** 2 **screening of spores of the filamentous fungus *Trichoderma reesei***

3  
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## 8 9 **Key words**

10 Flow cytometry; fungi; spores; kinetics; GFP; early stage growth indicators

## 11 12 **Highlights**

- 13 - Engineering of fluorescent spores of *T. reesei* expressing EGFP proteins through transformation
- 14 - Optimization of flow cytometry parameters for detecting early stage fluorescence expression
- 15 - Taking into account both growth and EGFP production as complementary signals
- 16 - Screening of nine promoters for fluorescence expression under FCM conditions: pTEF > pPKI >  
17 pGPD > pPDC.

## 18 19 **Abstract**

20 Flow cytometry (FCM) is a powerful technique still little used to study filamentous fungi due to  
21 physical constraints: the hyphae are too large to enter the FCM fluidic system, unless spores can be  
22 analyzed at a very early stage of germination. The technique nevertheless has strong potential for the  
23 study of these micro-organisms (spore sorting, viability, characterization etc.). This study focused on  
24 the investigation of several parameters, ranging from germination and storage conditions of *T. reesei*  
25 spores through to FCM gating, to detect their fluorescence during the first 24h of germination.  
26 Fluorescent spores were first obtained after aerobic germination at 25°C and monitored over 24h using  
27 FCM, to screen for nine promoters controlling a green fluorescent protein gene. The fluorescence  
28 signal (FL1) was then acquired, in addition to the growth characterization of the spores, based on the  
29 size signal or Forward Scatter (FSC). They were combined to identify the best candidate(s) from  
30 among the nine promoters for the strongest- and earliest-possible fluorescence emission, which

31 resulted in the following ranking: pTEF > pPKI > pGPD > pPDC. There are numerous possible  
32 applications of this work, ranging from molecular biology to monitoring fermentation.

33

## 34 **1. Introduction**

35 Micro-organisms produce various metabolites with industrial relevance. They display a high potential  
36 as biological alternatives to petroleum-derived fuels or chemicals such as ethanol (Bouws et al., 2008;  
37 Harman and Kubicek, 1998; Kumar et al., 2008). They often need to be improved through genetic  
38 engineering, which involves genetic transformation, *i.e.* the introduction of recombinant DNA in the  
39 cell nuclei and selection of the recombinant cells using selective markers. These should not be  
40 antibiotic resistance markers, commonly used in the lab for their efficiency and convenience of use,  
41 for safety reasons and due to concerns about horizontal gene transfers in the environment. Instead, if  
42 elimination of the marker is not possible or practical, auxotrophic or fluorescent markers must be used.  
43 Moreover, most selection, isolation and routine analyses in molecular biology optimization of micro-  
44 organisms are well-known methodologies. However, they remain very time consuming, since several  
45 weeks or at least days are required before the desired transformed strain can be identified, isolated and  
46 retrieved. Faster techniques have been developed to overcome these limitations, such as flow  
47 cytometry (FCM). This technique is a measure of physical and optical properties of cells transported  
48 by a liquid vector until a source of light excitation. The main advantage of FCM is to consider each  
49 cell individually, allowing an analysis based on quantitative as well as qualitative information. In 1934,  
50 Andrew Moldavan introduced the concept of cell counting using extinction signals when cells passed a  
51 photoelectric detector (Moldavan, 1934). The technique was improved by adding an injection system  
52 (Crosland-Taylor, 1953) and a laser as the light source (Van Dilla et al., 1969). Many optimizations  
53 were added later, and FCM became a key technique for the study of mammalian cells, bacteria and  
54 yeasts. The main advantages of this method are: 1) it is much faster than any other screening method,  
55 2) it is nondestructive, highly sensitive and multiparametric allowing simultaneous study of cell size,  
56 structure and fluorescence, and 3) with the addition of a sorter, cells that can be selected for further  
57 study (technology called Fluorescent Activated Cell Sorter or FACS, sorting a population in sub  
58 populations based on a labelling which can be fluorescence, size etc). However, FCM is not  
59 commonly applied to filamentous fungi due to a physical limitation: the mycelium growth mode.

60 Indeed, the size and structure of the filamentous mycelium prevent its analysis by flow cytometry due  
61 to the risk of obstructing the flow system, since the nozzle and sheath fluid diameter range from 30 to  
62 200  $\mu\text{m}$ , depending on the system. However, some devices do exist that allow the detection of cells up  
63 to 500  $\mu\text{m}$ . To the best of our knowledge, the limitation here is that these would not be compatible  
64 anymore with the high throughput needed for genetic engineering screening. Consequently, the cells  
65 studied may not exceed this threshold size. The presence of spores could also be considered a  
66 limitation, due to dissemination risks when different types of micro-organism are studied with the  
67 same device. Even so, the first study on the metabolic activity of a filamentous fungus, *Neurospora*  
68 *crassa*, was carried out using FCM in 1978, showing the potential of this application (Serna & Stadler,  
69 1978). Other studies were performed on several filamentous fungi such as *Trichoderma*, *Penicillium*,  
70 *Phoma* and *Candida*, focusing especially on characterization of viability, metabolic activity and  
71 morphology (Allman, 1992; Wenisch et al., 1997; Kullman, 2000; Bradner & Nevalainen, 2003; Peng  
72 et al., 2014; Vanhauteghem et al., 2017; Wen et al., 2020). While the combination of FCM and  
73 fluorescence efficiency was proven long ago for enumeration and fast isolation of bacterial and yeast  
74 cells, it was only recently that a study demonstrated the interest of this method for fungi, so long as the  
75 right conditions can be achieved (Bleichrodt & Read, 2018). Nevertheless, the use of FCM for fungal  
76 applications is still not possible for the mycelium, and thus focuses on spores. Spores exhibit very  
77 weak activity, meaning that only a short germination step will allow the flow cytometry technique to  
78 work properly. A proof of this concept was provided by Thronset et al. (2010), using a green  
79 fluorescent protein (EGFP, *i.e.* enhanced GFP) to select improved *T. reesei* strains through flow  
80 cytometry, at the stage of spore germination. The authors highlighted the importance of choosing the  
81 most relevant conditions for the expression of EGFP, since both strong and very early production was  
82 required for screening to work as expected. More recently, others have used FCM to screen *T. reesei*  
83 strains through overexpressed fluorescence, using spores (Gao et al., 2018) or protoplasts (Qin et al.,  
84 2018). These studies all placed fluorescence expression under the control of the *cbh1* gene promoter,  
85 well known for its strong induction of cellulase production in fungi. The drawback of this promoter is  
86 that it requires an induction step, which is usually done with the disaccharide sophorose, costly but  
87 effective since it leads to a both strong and early induction, or with lactose, leading to strong but  
88 slower induction, and therefore possibly incompatible with FCM requirements. To the best of our

89 knowledge, only one study tried promoters other than *cbh1* and proved that FCM can also be an  
90 interesting tool for evaluating promoter strength (Wang et al., 2018). Promoter strength is a key  
91 parameter in FCM, even if this is an indirect estimation. Indeed it would allow for easier and much  
92 more selective screening, given that there will be more separation between populations on cytograms.  
93 The aim of this study is to obtain a methodological guide for developing a fast and discriminant FCM  
94 approach for filamentous fungi. *Trichoderma reesei* was used as a model, for its industrial interest as a  
95 strong producer of cellulases. We first identified suitable promoters for EGFP production in  
96 *Trichoderma reesei*, under conditions compatible with the limitations of FCM, which means at the  
97 strongest- and earliest-possible stage. Then, the base-line fluorescence (*i.e.* auto-fluorescence of  
98 untransformed spores) was estimated for a control strain, allowing the design of some relevant gates of  
99 analysis, proposed in order to process the data obtained and to create qualitative and quantitative  
100 indicators. We also performed microscopy analysis in order to identify conditions compatible with  
101 limitations of our FCM, *i.e.* the growth of the spores in terms of size. Finally, we were able to  
102 distinguish between the fluorescence signal produced before and after induction and thus maybe  
103 encapsulated after transformation when collecting protoplasts, or resulting from a leaky promoter.  
104 Nine promoters were screened for fluorescence expression, which was monitored as a function of size  
105 distribution of the germinating spores over 24 hours. Growth and fluorescence production could be  
106 assessed separately but also combined and compared in order to identify the best candidate(s).

107

## 108 **2. Materials & Methods**

### 109 *2.1 Preparation of DNA vectors for transformation*

110 EGFP cDNA (Eurofins, Val Fleuri, Luxembourg) was amplified at a hybridization temperature of  
111 66°C with polymerase Q5 (New England Biolabs, Ipswich, Massachusetts, USA) using PCR. After  
112 purification (QIAquick PCR Purification kit, QIAGEN, Hilden, Germany), the products were  
113 submitted to digestion by the restriction enzymes *FspI* (5 U/μL) and *XhoI* (20 U/μL) (New England  
114 Biolabs, Ipswich, Massachusetts, USA).

115 To construct the vector, the plasmid pUT1140 was made up of a hygromycin-resistance, a replication  
116 origin from *E. coli* (pBR322), a *T. reesei cbh1* promoter (pCBHI) and a *T. reesei cbh1I* terminator  
117 (tCBHI). In order to insert the EGFP gene between the promoter and terminator, restriction enzymes

118 *FspI* (5 U/ $\mu$ L) and *XhoI* (20 U/ $\mu$ L) were used again for vector linearization. The two fragments were  
119 ligated using a DNA Ligation kit (Takara Bio Inc, Kusatsu, Shiga, Japan).  
120 After the ligation step, the vector was transformed in 10-beta *E. coli* (New England Biolabs, Ipswich,  
121 Massachusetts, USA) following the kit instructions. 100  $\mu$ L of the mix was spread on a petri dish  
122 containing LB Broth Lennox medium (Becton, Dickinson & Co) with 50  $\mu$ g.mL<sup>-1</sup> hygromycin  
123 antibiotic (Invivogen, Toulouse, France). Incubation was carried out at 37°C overnight.  
124 Colonies were transferred in liquid medium LB Broth Lennox with hygromycin and incubated  
125 overnight at 37°C under 180 rpm agitation. Cultures were finally centrifuged (5,000 g; 5 min). The  
126 pellets were recovered and submitted to QIAprep Spin MiniPrep Kit (QIAGEN, Hilden, Germany) in  
127 order to extract the plasmids, which were titrated with NanoDrop (ThermoFisher Scientific). The  
128 quality of the vector constructs was checked by electrophoresis after digestion using the same enzymes  
129 as before (*FspI* and *XhoI*): the presence of 2 bands at the expected lengths (one for the linearized  
130 plasmid and another for the insert) confirmed correct insertion.  
131 The *cbhI* promoter was then replaced in order to screen different promoters (pTEF, pTBP, pPTP, pPKI,  
132 pPDC, pHYD, pHFB, pGPD), using the same protocol and the same restriction enzymes *FspI* (5 U/ $\mu$ L)  
133 and *XbaI* (20 U/ $\mu$ L) (New England Biolabs, Ipswich, Massachusetts, USA).  
134 Finally, nine vectors containing EGFP were obtained under the different promoters.

135

## 136 2.2 *T. reesei* transformation process

137 The strain used as a reference is *T. reesei* CL847 (Durand et al., 1988), a close relative of the RUT  
138 C30 strain (ATCC® 56765<sup>TM</sup>, ATCC, Manassas, Virginia, USA). Transformation of the reporter gene,  
139 chosen as a green fluorescent protein or EGFP (enhanced GFP), was carried out using the protoplast  
140 transformation process (PEG/CaCl<sub>2</sub>) as described by Penttila et al. (1987). The final transformed  
141 strains were selected for resistance to hygromycin (50 $\mu$ g.mL<sup>-1</sup>).

142

## 143 2.3 Flow Cytometry acquisitions

144 All the analyses were carried out using a Cyflow Space cytometer (SYSMEX, Kobe, Japan), equipped  
145 with an MLS Blue 480-50 V2 laser emitting at 488 nm with a power of 50 mW. The optical detector  
146 range for FL1 was 520/20. Before analysis, the suspension of spores from a petri dish (9 cm) washed

147 with 5 mL of MilliQ sterile water, was diluted 1/40 in Mc Ilvaine Buffer (12.29 mL of citric acid (0.1  
 148 M) and 7.71 mL of Na<sub>2</sub>HPO<sub>4</sub> (0.2 M), at pH 4). All assays in FCM and quantifications were carried  
 149 out as technological triplicates on independent cultures (biological triplicates). The parameters of the  
 150 FCM were the following: speed 6, FSC 125, log3, SSC 200 log3, FL1 450 log4. All solutions of  
 151 spores were diluted to 1/40. All parameters were kept identical for all experiments.

152

153 **3. Results & Discussion**

154 3.1 Germination and storage conditions

155 A preliminary work was carried out to ensure that fluorescent spores from *T. reesei* could be obtained  
 156 for FCM characterization. The influence of several parameters was tested on the spore germination  
 157 process: growth medium, oxygen availability, agitation, light and temperature, as can be seen in

158 **Table 1.**

Parameter	Tested conditions	Description
Growth medium	Water vs Potato Dextrose medium	No germination in water, nutritive medium is required
Oxygen	Aerobic vs Anaerobic	No anaerobic germination, oxygen is required
Agitation	0 vs 150 rpm	No difference
Light	Day light vs obscurity	No difference
Temperature	4 vs 12 vs 20 vs 30°C	No germination at 4°C, little difference afterwards

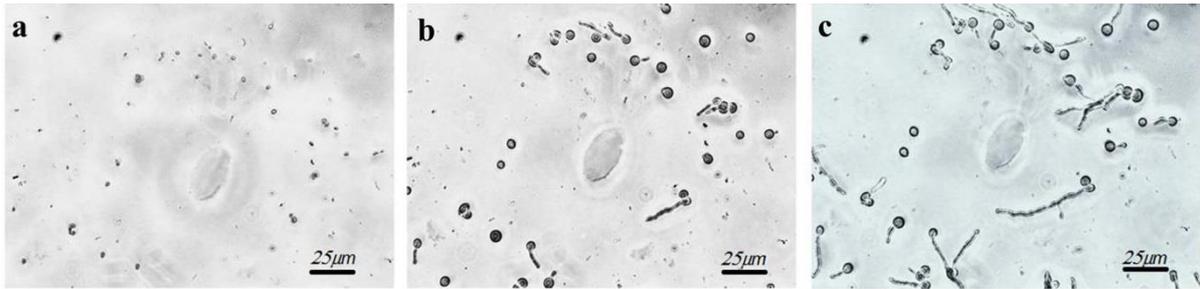
159  
 160  
 161  
 162

**Table 1: Comparison of environmental conditions on spore germination**

163 Storage conditions were therefore set as follows: 4°C in a nutritive medium PD (Potato Dextrose,  
 164 Becton, Dickinson & Co). The germination of the spores was then carried out at 25°C, in aerobic  
 165 conditions, under agitation at 150 rpm, still using nutritive medium PD. A specific medium was  
 166 required in the case of pCBHI, an inducible promoter, which was named F45 (8.7 g of K<sub>2</sub>HPO<sub>4</sub> + 4.2  
 167 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> + 0.3 g of MgSO<sub>4</sub> + 1.5 g of Corn Steep + 1 mL of trace elements + 11.6 g of malic  
 168 acid + 20 g of lactose = 1 L of F45) and adjusted to pH 6.

169 Once these optimal conditions were determined, the final step was to assess the germination kinetics  
 170 of the spores, to ensure they remained compatible with FCM. The germination process was thus  
 171 monitored over 37h and images were taken regularly and observed under light microscopy  
 172 (magnification x10). Representative images were chosen to illustrate the results, as can be seen in  
 173 Figure 1.

174

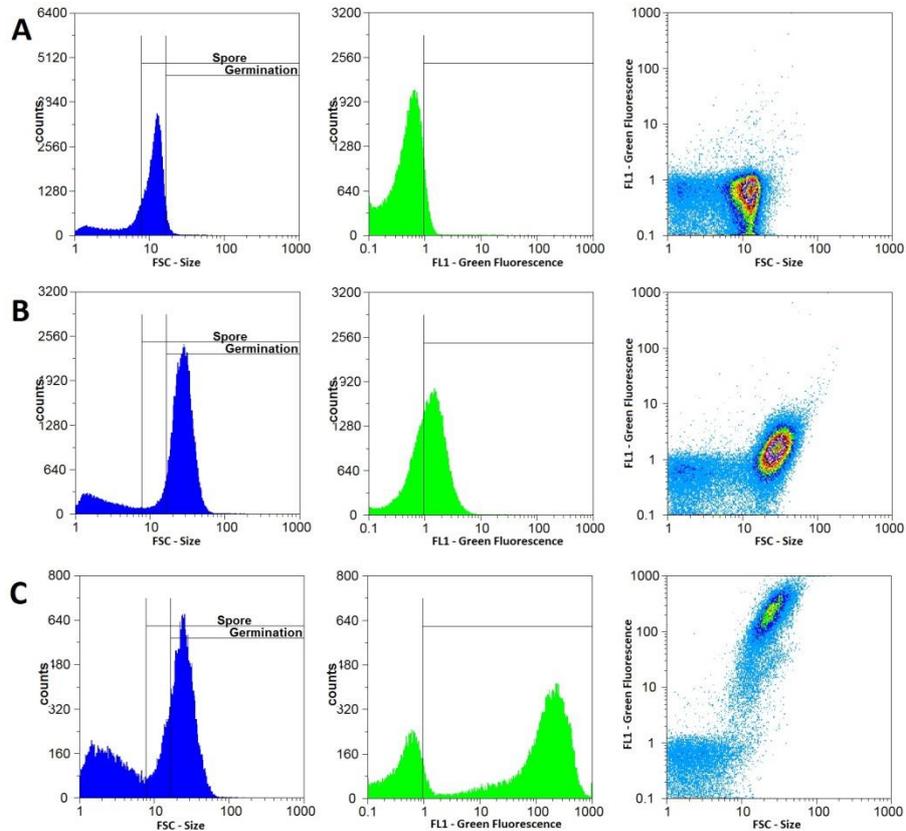


175  
176 **Figure 1: Germination of *T. reesei* spores monitored with light microscopy at (a) 0 hours, (b) 16 hours and (c) 20**  
177 **hours of culture. Magnification x10**

178  
179 At between 5h and 8h, swelling started with an increasing spore diameter (order of magnitude x2). At  
180 16h, hyphae appeared (Figure 1b) and at 20h, some branches were visible (Figure 1c). Finally, at 37h,  
181 a very dense mycelium was observed. It was concluded, therefore, that the spores must be  
182 characterized by FCM during the first 20h of germination.

183  
184 **3.2 Evaluation of CL847 autofluorescence and accurate design of the gating**

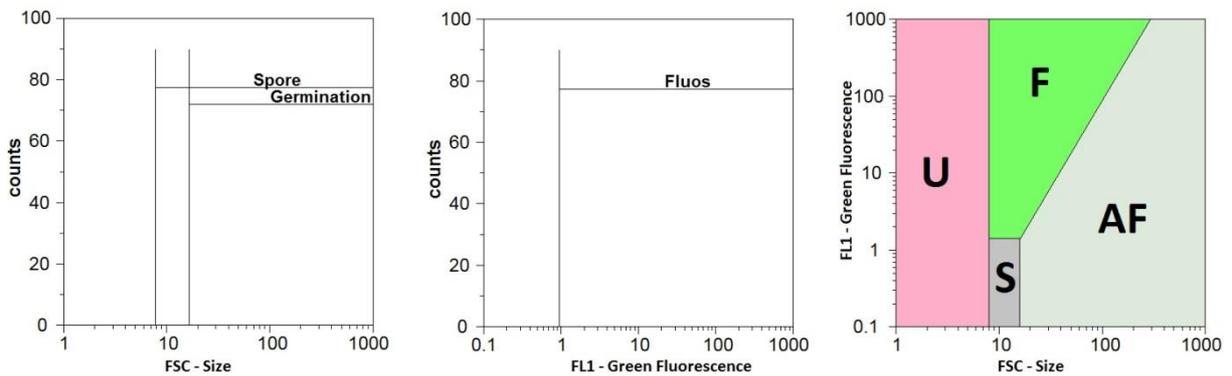
185 Once the germination conditions were properly assessed, spores were evaluated by FCM to determine  
186 the level of fluorescence that could be measured, allowing the design of a specific gating for analysis  
187 of the results (Figure 2).



188  
189 **Figure 2: Example of cytograms for untransformed control CL847 at t+0 (A) and t+24h (B) in PD rich medium;**  
190 **against transformed strain expressing EGFP at t+24h in PD rich medium.**

191  
 192 Fluorescence intensity was measured on ungerminated spores of the untransformed strain and spores  
 193 during their germination on PD rich medium. As can be seen in  
 194 Figure 2, this intensity increased with spore size and can be visualized from t0 (Fig. 2A) to t+24h (Fig.  
 195 2B) for CL847 as a linear profile between Fluorescence (FL1) and Size (FSC), which will be used as  
 196 the background reference. In contrast, when EGFP is expressed, the growth is similar to CL847 while  
 197 there is a major difference on FL1 axis, with a 3-log shift from Fig.2B to Fig.2C. This shows that there  
 198 will be no problem for distinguishing between autofluorescence (background) and actual fluorescence  
 199 expression due to EGFP.

200  
 201 Based on these results, a gating was proposed, as can be seen in Figure 3, in order to visualize any  
 202 emission of EGFP on FSC-FL1 cytograms.



203  
 204 **Figure 3: Acquisition gating for kinetic monitoring of fluorescence. Left = size distribution of the particles; middle =**  
 205 **fluorescence distribution of the particles; right = fluorescence vs size. Codes: S = ungerminated spore; F = spore**  
 206 **expressing GFP; AF = Auto-Fluorescent spore; U = Unsustainable spore.**

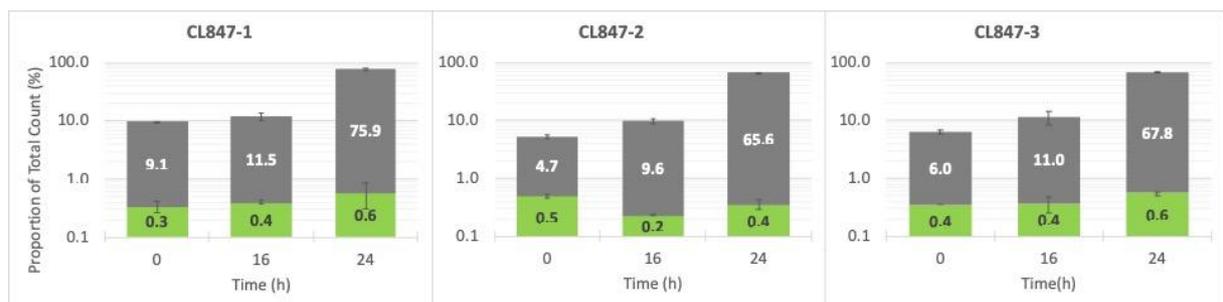
208 The AF gate was drawn based on the previous testing on CL847 (Figure 2B). Each event higher than  
 209 this background linear area was considered as corresponding to fluorescent cells of interest, which  
 210 were attributed to the F gate, as can be seen in Fig.2C. It can be noted that some spots appear on the  
 211 left, with lower FCS signal than the ungerminated spores (S gate), and the assumption was made that  
 212 these corresponded to cell debris and/or to particles from the medium (U gate). The assumption was  
 213 confirmed using double labelling IP/cFDA allowing identification of the dead cells (data not shown).  
 214 The S/F/AF gating thus provided a proper discrimination system between non-germinated (S),  
 215 fluorescent (F) and auto-fluorescent cells (AF).

216 Few studies describe the gating used for sorting cells, except for Wang et al. (2018) who focused on  
 217 the fluorescence activity for gating *T. reesei* spores or protoplasts. However, the authors did not  
 218 mention kinetic monitoring, which suggests that only one time point was analyzed. To the best of our  
 219 knowledge, studying the kinetics of the fluorescence emission of *T. reesei* with FCM as a function of  
 220 time has not previously been reported. Nevertheless, this is an important parameter to take into  
 221 account to avoid confusing two types of fluorescence. On the one hand, the real fluorescence produced  
 222 by GFP expression, on the other hand, the inner or auto-fluorescence resulting from growth (the bigger  
 223 a spore, the higher its fluorescence). It is thus interesting to go further into details about the present  
 224 gating, which is adapted for kinetic monitoring.

225

### 226 3.3 Kinetic monitoring and quantification of autofluorescence

227 The previously defined zones were applied to CL847 in a 24h time-course experiment.



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**Figure 4: Validation of the S/F/AF gating on the control strain CL847. Fluorescent cells expressing GFP (F) in green and Auto-fluorescent cells not expressing GFP (AF) in grey. Average values were obtained from a biological triplicate (CL847-1, CL847-2 and CL847-3) and technical triplicates (each strain was analyzed 3 times).**

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The proportion of the total count of the CL847 spores was plotted against time for the two populations F and AF according to the gating shown in Figure 3. The 100% value is never reached since cells found in the S area and/or in U area were not taken into account: a sharp decrease in the total count was found in the S area from t0 to t+24h. A biological triplicate was carried out, and for each CL847 (1, 2 and 3), a technical triplicate was also acquired (each of them were analyzed three times). As can be seen in Figure 4, we observed i) good reproducibility for the different CL847 cultures at each time point with variation coefficients of 4.8, 4.9 and 4.0% at t+24h for example; ii) the growth kinetic was effectively monitored, as increasing numbers of cells can be found in AF from t0 to t+24h (up to 70 to 76% which corresponds to 63.8, 48.6 and 58.5 k spots for the 3 biological replicates respectively); iii) very few cells were detected in the F gate (0.3 to 0.6%, which corresponds to 480, 310 and 380 spots

243 for the 3 biological replicates respectively) compared to the cells detected in the AF gate, especially at  
244 increased time points. The counts of spots occurring in the different areas can be compared as  
245 concentrations of spores, since the measurements were all carried out between two electrodes  
246 corresponding to 50  $\mu$ L. Moreover, the number of cells found in the F gate remained constant from t0  
247 to t+24h, suggesting that they were artefacts independent of cell growth. Considering that the artefacts  
248 were due to the definition of the gate and taking into account their very low percentages, we  
249 considered that this experiment allowed validation of the gating. These experiments also allowed its  
250 robustness to be tested.

251

#### 252 3.4 Screening promoters controlling EGFP expression in *T. reesei*

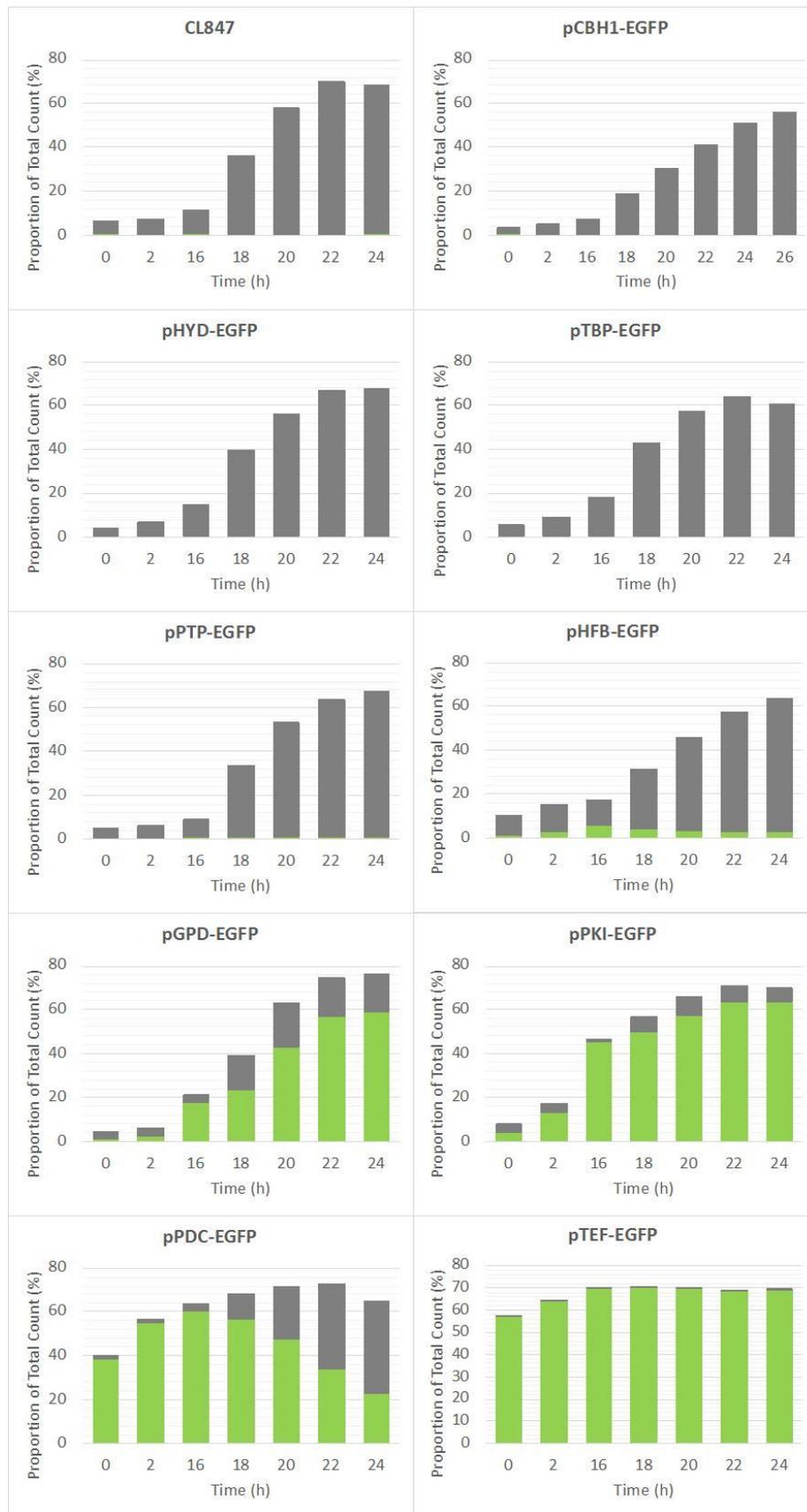
253 Nine promoters were screened in order to evaluate their strength and early expression using the EGFP  
254 fluorescence signal. The choice of the promoters was carried out based on RNAseq data available  
255 from *T. reesei* experiments in the lab (data not shown), providing the highest levels of fluorescence  
256 over the first 24h. The evolution of the fluorescence vs FSC signal of the detected cells was plotted  
257 over time (Figure 7, supplementary data).

258

259 CL847 and pPTP cytograms show a similar trend: cells moved from the S area to the AF area, without  
260 entering the F area which corresponds to growing spores without EGFP emission, or at least not a  
261 strong or early-stage emission. This suggests no or weak expression from the promoter. The same  
262 behavior was also observed for pCBH1, pTBP and pHYD (see supplementary data Fig.7). The result  
263 with pCBH1 may be surprising, as most of the literature dealing with *T. reesei* and FCM is based on  
264 pCBH1 controlling the expression of fluorescence. However, in these studies (Gao et al., 2018; Quin  
265 et al., 2018; Thronset et al. 2010), pCBH1 is induced by several days of growth in minimal media  
266 supplemented with lactose + sophorose, glucose + sophorose or lactose only. Here, we not only used a  
267 different inducer (F45 medium), but also used a very short induction time, explaining the apparent  
268 discrepancies. For four other promoters, as exemplified in Figure 5 by pPKI and pTEF, the cell  
269 population moved from the S area towards the F area in 18h, with 50% and 70.3% of the total count  
270 found in the F area respectively. Even if a small sub-population of cells could be found in the AF area,  
271 strong and early-stage fluorescence was observed and demonstrated good potential for compatibility

272 with FCM analysis. This trend was typical for pPKI and pTEF and to a lesser extent for pGPD and  
273 pPDC. The latter were intermediates, as the cell population for these two promoters was equally  
274 dispersed between the F and AF areas, suggesting weaker expression. These results are in good  
275 accordance with Wang et al. (2018) who also found a strong expression of GFP in *T. reesei* spores  
276 using FCM for the following promoters: pTEF slightly > pPKI >> pPDC. To the best of our  
277 knowledge, the latter was the only study in the literature that tried several promoters for better  
278 expressing fluorescence in order to be compatible with FCM requirements for screening early  
279 transformants. Based on the present results, a ranking of the promoters could be determined as follows,  
280 based on observation of the cytograms: pTEF > pPKI > pGPD > pPDC > pPTP, pHYD, pTBP, pHFB,  
281 pCBH1.

282 However, it should be noted that pTEF and to a lesser extent pPDC, displayed atypical behavior, in  
283 that the cells were already located in the F area at t<sub>0</sub>, showing that some fluorescence must have been  
284 encapsulated during sporulation. Indeed, after transformation, the strains were selected on a rich  
285 medium (with antibiotic), which allowed them to produce some EGFP during their growth. pPTEF and  
286 pPDC were probably induced during or before sporulation. This was checked under confocal laser  
287 scanning microscopy where mycelium was indeed fluorescent before sporulation occurred (data not  
288 shown). Two considerations drove the next step of the study, focusing on the fluorescence intensities: i)  
289 the encapsulation of fluorescence mentioned above; and ii) the artefact of fluorescence mentioned in  
290 3.1, which required comparison of the intensities to determine if the chosen promoter provided higher  
291 fluorescence. This objective was reached by isolating the F and AF cells and plotting the evolution  
292 over time of the proportion of total count in both areas, as can be seen in Figure 5.



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**Figure 5: Evolution over time of the proportion of total count in S (green) and AF (grey) areas for the nine screened promoters and the control CL847. Averaged values from triplicates.**

297 To the best of our knowledge, this is the first time that quantification of FCM signals has been  
298 assessed this way, for *T. reesei* or any other filamentous fungus. Indeed, our understanding of  
299 literature was that the studies aimed for the determination of the viability of spores and metabolic  
300 activity (Peng et al., 2014; Ehgartner et al., 2016; Vanhauteghem et al., 2017). In the present study, we  
301 succeeded in decorrelating the fluorescence coming from the expressed protein (EGFP) from the inner  
302 fluorescence coming from the growth (the bigger a spore, the higher its volume and autofluorescence).  
303 Firstly, fluorescence production (F area) could be quantified for pTEF, pPDC, pPKI and pGPD, as  
304 expected. A very low amount of fluorescence was detected as F for pHFB, but it remained at baseline  
305 level over time. Secondly, the fluorescence production profiles were very different between pTEF,  
306 pPDC, pPKI and pGPD. One common pattern for pTEF and pPDC was that some fluorescence was  
307 already detected at t0. It was a high fluorescence intensity, since 38% and 57% of the cells were  
308 already located in the F area for pPDC and pTEF respectively. Conversely, pPKI and pGPD displayed  
309 very low EGFP levels at t0, but they both led to high EGFP expression at t+22h and t+24h, with  
310 respectively 63% and 58% of the total cell count located in the F area at these final stages. Finally, it  
311 should be noted that pPDC is atypical in terms of fluorescence emission over time, as it seems to  
312 decrease from t+16h to t+24h. This could be a negative aspect of using this promoter, since the growth  
313 of the spores could happen faster than EGFP production, leading to a “dilution” of the fluorescence  
314 signal. As a consequence, the final step for truly discriminating promoters and choosing the best  
315 candidate(s) will be to find the right balance between the kinetics of EGFP production and spore  
316 growth.

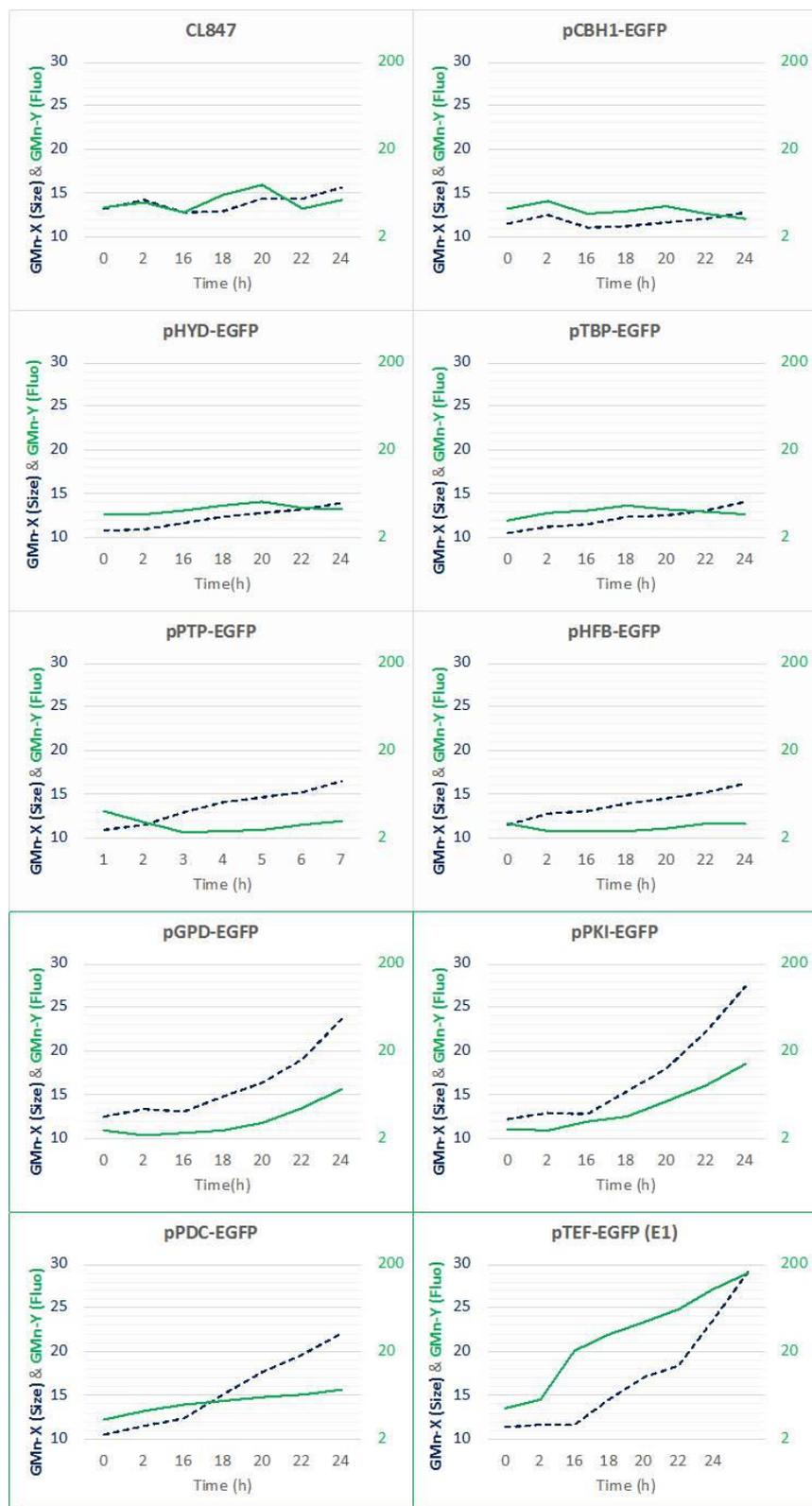
317

### 318 3.5 Deconvolution of growth and GFP expression

319 This final objective is important in order to avoid choosing the wrong promoter based only on  
320 fluorescence intensity. In the best-case scenario, fluorescence production and growth should be clearly  
321 discriminated. Looking deeper into each population will avoid relying only on the gating (compulsory  
322 for cell sorting), by also providing quantified data that can be retrieved, compared and submitted to a  
323 significance test.

324 From this perspective, the proportion of counts in the F and AF areas were no longer sufficient. It was  
325 necessary to isolate the fluorescence from the growth, which is why the evolution of the geometric

326 average value of size (GMn-X) was plotted against time, as was the geometric average value of  
 327 fluorescence (GMn-Y), as can be seen in Figure 6. Both signals were quantified from the cells located  
 328 in the F area, in order to access the fluorescence intensity reached in F only.



329  
 330 **Figure 6: Evolution overtime of the size (GMn-X, blue) and the fluorescence (GMn-Y, green and log scale) signals**  
 331 **taken from F cells for the nine screening promoters and the control CL847. Average values from triplicates.**

332

333 Figure 6 shows that pHYD and pTBP were no different from CL847. A small proportion of cells was  
334 located in the F area for these three strains (see Figure 5), and these cells were obviously neither  
335 growing nor emitting any fluorescence. Then pPTP and pHFB displayed similar behavior, also close to  
336 the control CL847. However, since some more cells (1% and 2.6% respectively) were detected in the  
337 F area for pPTP and pHFB, growth was faster than for the previous two strains and the control.  
338 However, here again, no fluorescence emission could be found over time. This example was the  
339 perfect illustration of how Figure 5 and Figure 6 are complementary and should be compared. Indeed,  
340 on the one hand, Figure 5 represents the proportions of spores found in F and AF areas. It gives a first  
341 comparison of the promoters, however at this stage it is almost binary: either a promoter is interesting  
342 or not. On the other hand, Figure 6 focuses on F area, giving the geometric average values of  
343 fluorescence, thus the intensities. Only here we can properly rank the promoters which seemed  
344 promising from Figure 5. This was the case for the four promoters pGPD, pPKI, pPDC and pTEF,  
345 which were strongly different from the control. Another interesting example is the comparison  
346 between pPKI and pTEF. When looking at the size, the growth appeared very similar. However, the  
347 fluorescence intensity was ten times higher for pTEF than pPKI at t+24h. Here again, Fig.6 is essential  
348 since looking at the counts in the different gates does not give the same information. Indeed, on Fig.5,  
349 it seems that pPKI and pTEF are of comparable interest at t+24h, while it was just shown thanks to  
350 Fig.6 than pTEF is the most promising one.

351 As a consequence, pTEF is the promoter that stood out the most: it not only expressed the highest  
352 levels of EGFP from t0 to t+24h, but the kinetic of EGFP production was also the fastest and strongest.  
353 All graphs being at the same scale, it is clear from Figure 6 that pTEF is the only promoter for which  
354 the EGFP production was higher than the growth of the spores over time. It should be specified that at  
355 this stage we did not take into account the insertion sites or the number of copies, which is ongoing  
356 work in order to assess a clone effect. The most relevant information at this stage was thus the timing  
357 of the fluorescence emission.

358 The discrimination of growth and fluorescence signals allowed for finer and more rigorous ranking of  
359 the promoters: pTEF > pPKI > pGPD > pPDC. Indeed, for a maximum level of EGFP, pTEF is the  
360 best candidate. Since some fluorescence encapsulation obviously occurred with pTEF, this selection is

361 based on the kinetic of EGFP production (highest slope of the experimental green curve for pTEF in  
362 Figure 6).

363

#### 364 **4. Conclusion**

365 Methodologically speaking, this study allowed determination of good conditions for germination and  
366 storage of spores, the correct parameters for application of FCM to filamentous fungi such as *T. reesei*  
367 and gating design for the identification of the fluorescent population separately from the baseline and  
368 native autofluorescence of the spores. Thanks to this combination of good practices, it was shown that  
369 FCM represents a valuable tool for screening promoters based on fluorescence detection, under  
370 conditions coupling of early stages of growth with a strong level of EGFP expression. Moreover, this  
371 work allowed i) ranking of the best candidate(s) for strong and early-stage EGFP production among  
372 nine promoters, ii) discussion of relevant indicators in order to discriminate both production of the  
373 protein of interest (here, EGFP) and the growth of the strain (biomass). The total number of spores, the  
374 number of germinated spores and the number of spores expressing EGFP could all be quantified using  
375 our method. On the other hand, a promising indicator can still be investigated based on the  
376 complementarity of fluorescence and size determinations in order to find the best compromise  
377 between production and growth. It would be an interesting way to directly estimate the specific  
378 productivity of fluorescence (or any kind of protein) over biomass production. It can most certainly be  
379 optimized even further based on a reference size, a given time of interest, etc. Modeling the  
380 relationship between the protein of interest/total protein and protein of interest/biomass, by following a  
381 batch over fermentation using FCM, would allow prediction of the productivity of a given strain for a  
382 given process without going through the tedious screening of clones. This work thus points towards  
383 interesting leads to exploit the full potential of FCM, going further than only viability indicators or  
384 raw fluorescence monitoring, which are the parameters most studied today.

385

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391

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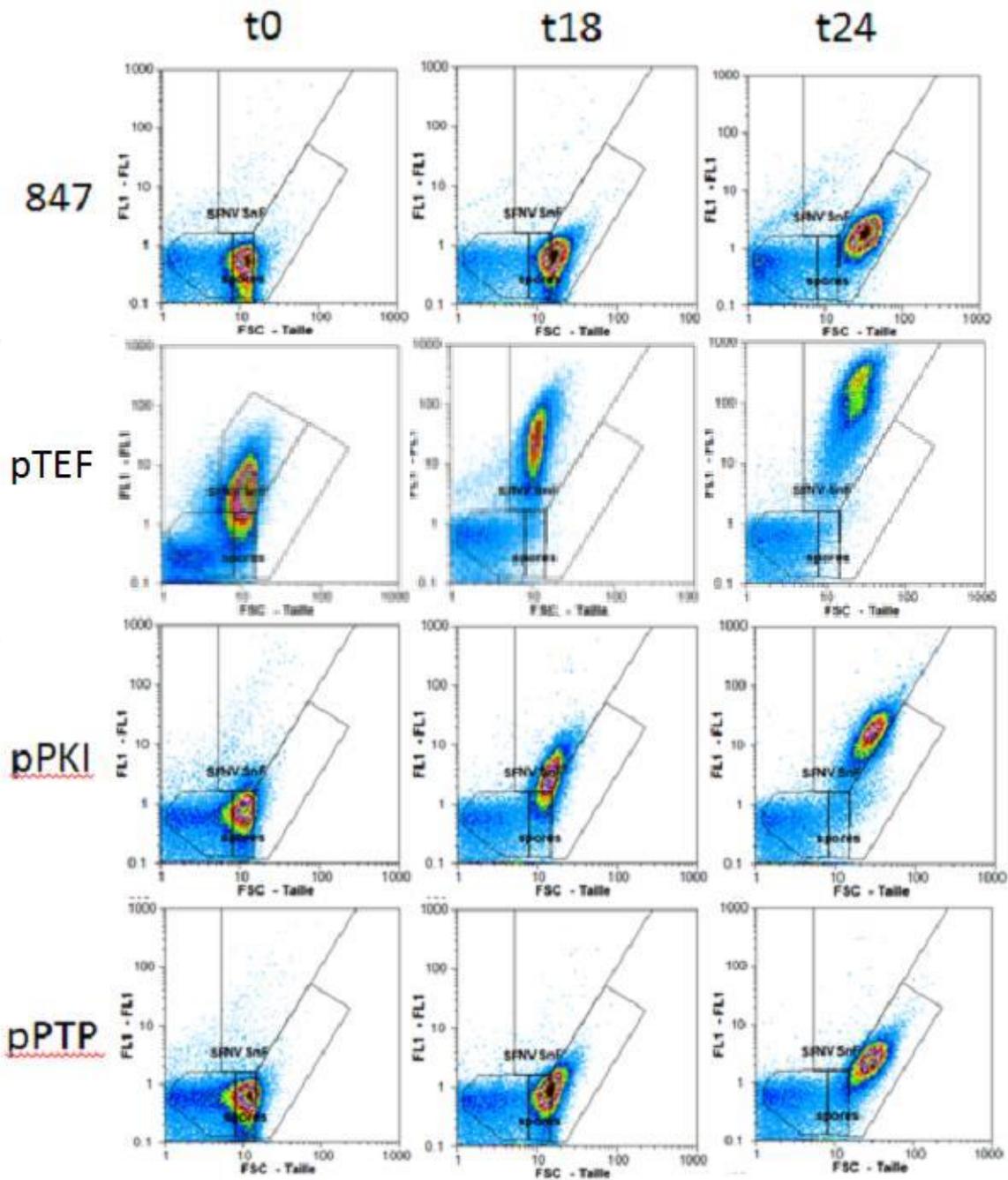
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Supplementary material



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Figure 7: Evolution of the profiles giving fluorescence (FL1) = f(size, FSC) for control CL847, pPTP-EGFP, pPKI-EGFP, pTEF-EGFP at t0, t+18h and t+24h.

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