1   **Monitoring of microalgal cultivations with on-line, flow-through microscopy**

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16

17 **Abstract**

18 Microalgal cultivations present challenges for monitoring and process control posed by their

19 large scale and the likelihood that they will be composed of multiple species. Cell

20 concentration is a fundamental parameter in any cultivation but is typically performed using

21 off-line methods that may be time-consuming, laborious, or subject to interferences. Here, an

22 in-situ microscope has been adapted to monitoring microalgal cultivations by adding a flow-

23 through cell and adjusting image-processing algorithms. After installation in the bypass of a

24 photobioreactor, the microscope enabled the continuous, automated acquisition of cell

25 count, cell size, and cell morphology data on-line during cultivation processes over a period

26 of 20 days, without sampling. The flow-through microscope was tested in cultivations of

27 *Chlamydomonas reinhardtii* and *Chlorella vulgaris.* Cell concentration measurements were in

28 agreement with off-line optical density measurements for both species. In addition, cell size

29 and morphology distributions were obtained that revealed population shifts during the

30 cultivation of *C. vulgaris*. This monitoring system thus provides a means to obtain detailed,

31 non-invasive insights of microalgal cultivation processes.

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34 Keywords

35 Flow-through microscopy, microalgae, automated image processing, cell count, cell size

36 distribution

37

1. Introduction

The industrial cultivation of microalgae has been the focus of increased attention in recent years, although the first commercial operations date to the 1960s. Various microalgae are cultivated to produce food, food supplements, pigments, and lipids for conversion to biofuels. Microalgae are usually cultivated in batch or semi-batch processes with cultivation times of up to 20 days. The monitoring of microalgal cultivations is of importance for process control. Of particular interest are parameters related to the biological system, including cell concentration and composition, which provide information about the status of the cultivation. Data on cell morphology and size are useful for monitoring the presence of contaminating species and the health of the culture. Ideally, these measurements would be made continuously, with the sensor system interfaced with the photobioreactor or pond in either an in-situ or an on-line format [1].

One approach to this goal is the use of continuous, non-invasive microscopic monitoring. The first in-situ microscope (ISM) was developed in 1990 [2] and has since been improved by several researchers [3]. It has been employed for the in-situ monitoring of yeast, mammalian, and microcarrier cultivations as well as crystallization processes of amino acids, proteins, and pharmaceuticals [4]. Most ISM systems are based on a transmitted light microscope and can be mounted in a 25-mm side port of a bioreactor. The sampling zone is thus immersed in the cultivation medium. Images of microorganisms or crystals are acquired and processed using particle-specific algorithms, yielding estimates of several parameters, including particle count, size and morphology.

The goal of this project was to develop and evaluate the ISM strategy for monitoring microalgal cultivations. Cultivations of two algal species were conducted and compared with standard off-line measurements of optical density.

2. Materials and Methods

2.1. Flow-through microscope hardware

The flow-through microscope (FTM) is a modified version of the in-situ microscope ISM III XTF (Sartorius Stedim Biotech GmbH, Göttingen, Germany) [5]. It has been used successfully for monitoring of various biotechnological processes such as cultivations of microorganisms and protein crystallization. The ISM III XTF is a transmitted light, bright-field microscope that can be mounted in a 25-mm side port of a bioreactor.

For the monitoring of microalgal cultivations, several modifications of the ISM III XTF were necessary to allow removal of adhered cells within the measuring zone, provide a replaceable light source, and integrate the device into the glass tubular photobioreactor used in this study. To accomplish these goals, the outer tube of the ISM was redesigned by replacing the sampling zone with a flow cell and by adding inlet and outlet metal tubes that can be integrated into the bypass of a photobioreactor. The upper segment of the microscope containing camera, objective lens, and motors was mounted on the outer tube (Fig. 1). Since the whole system has a modular construction, individual parts could be replaced easily. A white LED was attached on the other side of the flow cell (Fig. 1). This construction allowed the flushing of the microscope windows by temporarily increasing the flow rate through the flow cell.

2.2. Image analysis and hardware control software

It was also necessary to alter the image-processing algorithms of the ISM III XTF to recognize microalgal cells. The original instrument software includes the control program InSitu Control for controlling the ISM hardware and camera parameters and for recording microscope images, as well as the image processing software InSitu Analysis for performing cell recognition and to allow parameter computation either on- or off-line. The algorithms for image processing for cell detection were originally designed for yeast cells and were based on border-tracking methods. Differences of grey values to the mode value (image

4

94 background) were used to define the border of possible cell objects. Since algae images

95 tended to be noisier, and consequently produced blurry object borders, this led to processing

96 artifacts in this application to algal cells.

97

98 Modification of the software consisted of migration from Delphi to C#, and implementing the

99 SUSAN procedure [6] for border tracking and cell detection to allow better object recognition

100 from noisy and out-of-focus images. Furthermore, the "Cell Wiper" function was introduced

101 to enable recognition of cells that have stuck to the sampling zone surface. These adhered

102 cells could be then excluded from counting. For each microalgal strain, additional strain-

103 specific algorithm optimization was necessary to account for different cell shapes.

104

105 The resulting software is capable of computing three process variables as primary

106 information: cell count in an image, and cell size and cell eccentricity for each cell identified

107 in the image. Furthermore, the software produces other information from these primary

108 data: cell volume of individual cells, total cell volume in a given liquid volume (biomass

109 concentration), detection of double cells and cell clusters, and classification into large,

110 medium, and small cells. Processing parameters and all results for each image are recorded

111 in a separate file linked to the image. From these files, all data can be exported, for each cell

112 and each image individually or as a summary, into a .csv file. Cell size distributions can also

113 be exported as a histogram. All variables are visualized in the GUI in real time.

114

115 *2.3. Microscope calibration*

116 For absolute cell area determination, the microscope-camera system was calibrated. A film

117 with a microscale was inserted into the measuring zone and pixels over a distance were

118 counted manually in the image. The area of one pixel was computed from pixel count per

119 micrometer. When the 10X microscope objective was used, a pixel had an area of 0.67 μm^2,

120 while the 20X objective yielded a conversion of 0.17 μm^2 per pixel.

121

2.4. Photobioreactor system

Two photobioreactor (PBR) cultivation systems were used for testing the FTM. Both are based on glass tubes of 45-cm length and outside diameter of 8 cm (volume 1.9 L) equipped with two tubing connections and an inner glass tube of 2.4-cm inside diameter placed along the centerline. The light source was an Osram 640 13W Universal White fluorescent lamp that was located in the inner glass tube and provided illumination with average intensity (PAR) of 58 µmol s^{-1} m^{-2} as measured 3 cm from the lamp surface. To accommodate the aeration, cooling, and sensors, two variants of a monitoring/addition vessel (MAV) were employed, both with a working volume of about 0.5 L. The first MAV was a glass vessel equipped with sensor ports in the lid for temperature, pH, and pO_2, and with a gas inlet and outlet. The second MAV was a steel double-jacketed unit with an inoculation port, a mixer, ports for temperature and pH sensors, and connections for gas inlet and outlet. In each experiment, one of these MAVs was attached to the glass tube by flexible tubing, and a peristaltic pump was used to circulate the growth medium between the glass tube and the MAV. Gas sparging took place only in the MAV. The first system, PBR-1, consisted of two glass tubes with the glass MAV and had a total volume of 4.4 L, while PBR-2 consisted of a single glass tube and the stainless steel MAV; this system had a total volume of 2.4 L. In PBR-1, temperature and pH were measured but not controlled, whereas temperature and pH were controlled by a Sartorius control unit Biostat B in PBR-2. In all experiments, CO_2 was supplied by sparging the liquid in the MAV with a mixture of 3% CO_2 in air at 1 vvm. The FTM was placed in the PBR bypass and supplied with cell suspension using a second peristaltic pump (Fig. 2).

2.5. Algal cultures, experimental conditions, and measurements

For all cultivation experiments, axenic cultures under sterile conditions were employed. TAP medium [7] was used for all cultivations. On-line cell measurements using the flow-through microscope were evaluated with the modified InSitu Control and InSitu Analysis software.

150   A set of four cultivations of *Chlamydomonas reinhardtii* (strain SAG 33.89, SAG Culture

151   Collection, Göttingen, Germany) was performed in the PBR-1 system at approximately 26 °C

152   and pH 7. *C. reinhardtii* is a green microalga with approximately spherical shape and a

153   diameter of 14 to 22 μm. Cultivations were inoculated with a 100-mL culture of *C. reinhardtii*

154   cells grown for 10 days in an illuminated shake flask. The optical density (OD) during the

155   cultivation experiments was measured off-line at 550 nm approximately every 24 h using a

156   Uvikon spectrophotometer. A 10X objective was used in the flow-through microscope. The

157   on-line cell count was computed as cell count/image. Image acquisition and evaluation was

158   performed in cycles every hour, each cycle comprising 100 images in 1-s interval.

159

160   A second set of two cultivations was carried out using *Chlorella vulgaris*, a green microalga

161   with spherical shape and a diameter of 4 to 10 μm. The cultivation was performed in the

162   PBR-2 system at 26 °C and pH 7, inoculated with a 100-mL culture of *C. vulgaris* cells grown

163   for 10 days in an illuminated shake flask. The optical density was measured at 750 nm,

164   outside the absorption range of both chlorophyll a and chlorophyll b, to avoid interference by

165   variable chlorophyll content. As *Chlorella* cells are smaller, a 20X objective was used in the

166   flow-through microscope. The on-line cell count was measured as in the *C. reinhardtii*

167   cultivation and computed also as cell count/image.

168

169

170   **3. Results and Discussion**

171   **3.1. C. reinhardtii *cultivation monitoring***

172   For the initial evaluation of the FTM for algal cultivation monitoring, cell number

173   concentrations were computed from the analysis of images acquired by the FTM during the

174   *C. reinhardtii* cultivations. Figure 3 is a comparison of these FTM-derived data with the off-

175   line OD measurements at 550 nm. Although the data computed from the images of the flow-

176   through microscope became noisier after 300 h of cultivation, filtering using a 12-h

177   asymmetric median was successful in providing a smoother output. Another comparison of

7

178   the on-line FTM data and the off-line OD measurements was the calculation of the ratio of

179   the off-line optical density and the median of the cell count obtained from the flow-through

180   microscope data. Ideally, this ratio should be constant during the cultivation no matter the

181   dimensioning of both variables. In these measurements, the relative difference between the

182   two methods decreased with increasing cell concentration (Supplemental Data Figure S-1).

183

184   **3.2. C. vulgaris *cultivation monitoring***

185   Based on experiences gained during the analysis of the first set of experiments, the data

186   collected by the FTM during the *C. vulgaris* cultivations were analyzed using several

187   additional methods. To account for the presence of a small number of clusters of cells that

188   might skew the data, information from the cell size and eccentricity data collected by the

189   FTM were used to create a function for elimination of likely cell clusters. FTM images from

190   the early growth phase were manually evaluated to determine the parameters of this

191   function, which is shown in Supplemental Data Figure S-2. Cell clusters were screened as

192   follows: (a) all objects larger than 1400 pixels were assumed to be clusters, (b) all objects

193   smaller than 600 pixels were assumed to be single cells, and (c) for objects in the size range

194   600 – 1400 pixels, objects below the corresponding critical eccentricity for a given pixel size

195   (Supplemental Data Figure S-2) were assumed to be single cells. This screening process

196   resulted in the elimination of 3.6% of the images in the early and late growth phases, and 4%

197   of the images in the stationary phase. These procedures yielded a dataset containing all

198   cells identified in the images acquired during the whole cultivation, with every cell linked to

199   its size and eccentricity, with cell clusters eliminated. All subsequent analyses resulting in

200   cell size and eccentricity distributions were carried out using individual cells and their

201   individual parameters.

202

203   These corrected, individual cell data were used to determine cell number concentrations in

204   1-h cycles, and these 1-h data were smoothed using a 12-h asymmetric moving average

205   filter to compare them to the off-line OD measurements (Fig. 4). As with the *C. reinhardtii*

cultivations, the on-line data were in good agreement with the off-line OD measurements,

although the FTM values were somewhat lower near the start of the experiment (until about

165 h). Three growth phases were delineated: early growth (94–179 h), late growth (180–

263 h), and stationary (264-335 h).

Cell size distributions were computed from the corrected FTM data separately for each of the

three growth phases (Figure 5). A substantial shift in the size distribution between the early

growth and the later two stages can be observed by comparing average and median cell

sizes for each stage, computed from the dataset containing all cells detected in FTM images.

Average cell sizes in the early, late and stationary phases were 77, 65 and 65 μm^2,

respectively, and median cell sizes were 71, 61 and 62 μm^2, respectively. Specifically, the

cell population became smaller on average after the first 180 h because of the loss of larger

(90-160 μm^2) cells. Distributions of cell size in bar chart format are shown in Supplemental

Data Figure S-3. Using off-line measurements in a Coulter Counter, Work et al. [8]

determined that *C. reinhardtii* CC124 cells grown in nitrogen-replete medium (corresponding

to the early growth phase) were 13% larger than cells grown in nitrogen-deficient medium

(corresponding to late growth and stationary phases). This shift in size distribution could

explain the differences between the OD and FTM measurements between 100 and 200 h.

Similarly, distributions of the cell morphology, expressed as eccentricity, were obtained from

the *C. vulgaris* dataset, resulting from the analysis of FTM images corrected for presence of

clusters. Distributions of eccentricity data in each of the three cultivation phases are shown

in Figure 6, and distributions of eccentricity in bar chart format are shown in Supplemental

Data Figure S-4. During the early growth phase, the *C. vulgaris* cells were less elongated

than those later in the cultivation.

3.3. General aspects of in-situ, flow-through microscopy of microalgal cultivations

Although microalgal cell concentration data can be obtained off-line using flow cytometry,

9

234   Coulter Counters, and optical absorbance, the results presented here are the first reports of

235   on-line, automated, continuous measurement of microalgal cell concentration. While other

236   methods such as optical absorbance can be modified for on-line application, the need to

237   provide dilution at higher cell concentrations, to avoid wavelengths affected by pigments,

238   and to correct for changes in cell size and composition (e.g., the presence of lipid bodies)

239   has proven challenging. Using FTM, these issues can be avoided, and additional

240   measurements such as the distributions of cell size and cell shape can be obtained.

241   Although shear stress from pumping the cells through the bypass may be a concern for

242   some microalgae, there was no discernible impact for the two species used in these

243   experiments.

244

245   Continuous measurements of cell concentration and cell size distribution have many

246   potential uses in large-scale microalgal cultivations. Nutrient addition and harvesting

247   strategies could be triggered when a particular cell concentration is reached, corrective

248   actions could be taken if the growth rate is not as expected, or the impact of predators (e.g.,

249   rotifers) could be detected at an early and potentially correctable stage. The additional

250   information content of continuous cell size distribution measurements could also provide

251   evidence of lipid accumulation (if associated with cell size increase) and the presence of

252   non-target microbial species. Those goals would be aided by modification of the image-

253   analysis software to provide data on the population morphology.

254

255   The modifications to the commercial ISM described here allow the imaging zone to be

256   cleaned automatically in the event of moderate cell adhesion. If more severe cell

257   accumulation is encountered, the microscope can easily be disconnected, cleaned, and

258   connected again under sterile conditions. Moreover, different light sources can be used with

259   simple replacement.

260

261 The results reported here demonstrate the basic capabilities of this system for obtaining on-

262 line data during algal cultivations. Such a system can enable the collection of a high density

263 of information about cultivations in laboratory and commercial systems, potentially leading to

264 new insights into the basic and applied biology of algae.

265

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267 **References**

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291

292

293 **Figure captions**

294

295 Figure 1. Schematic of the flow-through microscope. Inset: detail of measuring zone.

296

297 Figure 2. Schematic of the flow-through microscope placement in the photobioreactor

298   bypass.

299

300 Figure 3. Comparison of off-line cell density measurements (OD) with data computed from

301   in-situ FTM analysis during a cultivation of *Chlamydomonas reinhardtii*. Raw data were

302   smoothed with a 12-h asymmetric median filter.

303

304 Figure 4. Comparison of off-line cell density measurements (OD_{750}) with data computed

305   from in-situ FTM analysis during a cultivation of *Chlorella vulgaris.* Cell count data were

306   acquired and computed every hour, and were smoothed by a 12 h asymmetric moving

307   average filter.

308

309 Figure 5. Cell size distributions in different phases of a *C. vulgaris* cultivation in PBR-2 as

310   measured by the FTM. Phase 1 is the early growth phase (94-179 h), Phase 2 is the

311   late growth phase (180-263 h), and Phase 3 is the stationary phase (264-335 h). Data

312   resulting from cell clusters were removed using the experimentally derived function

313   described in the text.

314

315 Figure 6. Distributions of *C. vulgaris* morphology in terms of eccentricity during different

316   phases of a *C. vulgaris* cultivation in PBR-2 as measured by the FTM. Eccentricity is

317   defined such that a value of 1.0 corresponds to a circle and larger values are ellipses.

318   Phase 1 is the early growth phase (94-179 h), Phase 2 is the late growth phase (180-

319   263 h), and Phase 3 is the stationary phase (264-335 h). Data resulting from cell

320   clusters were removed using the experimentally derived function described in the text.

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7   **Acknowledgments**

11

12

Figure

upper segment

lower segment
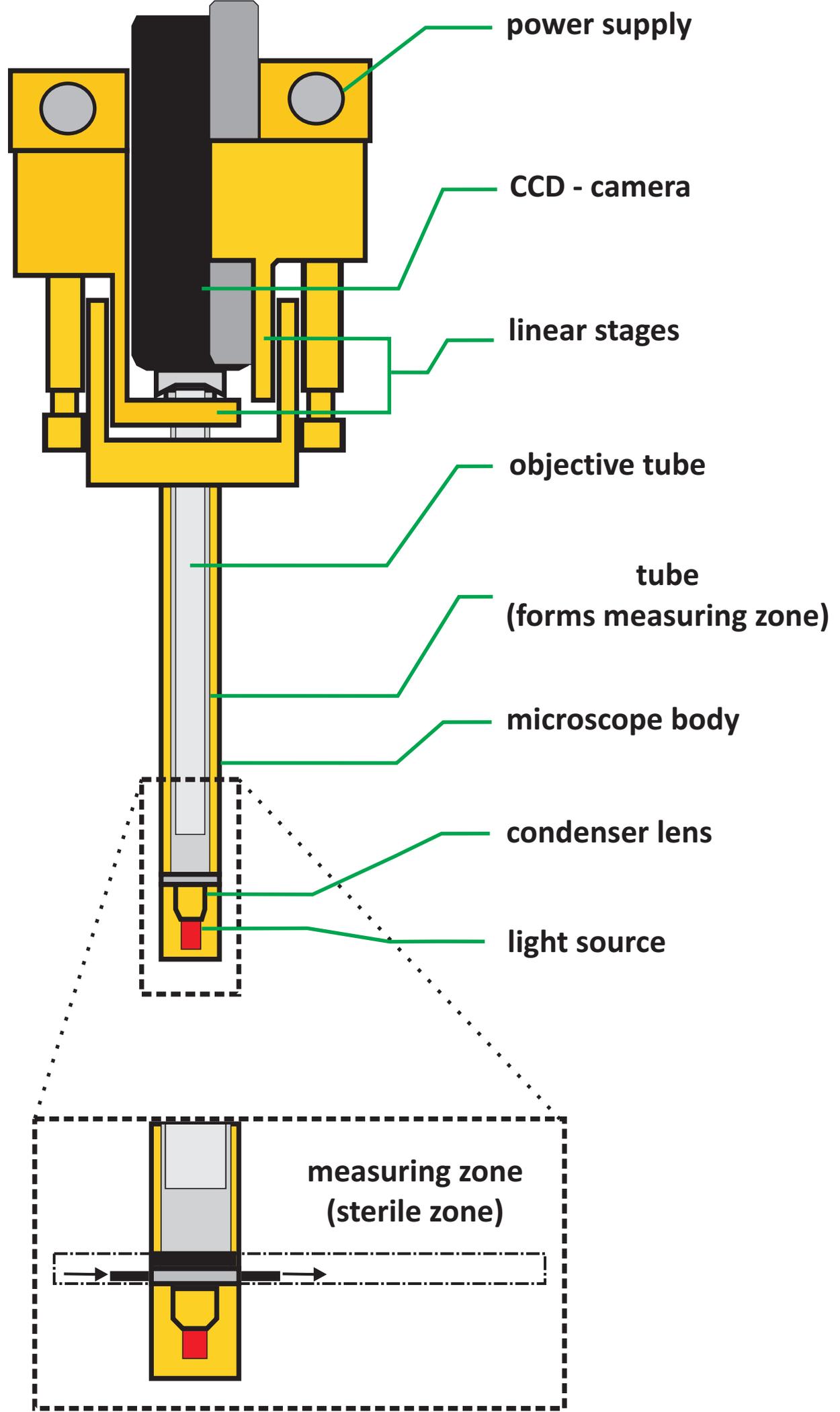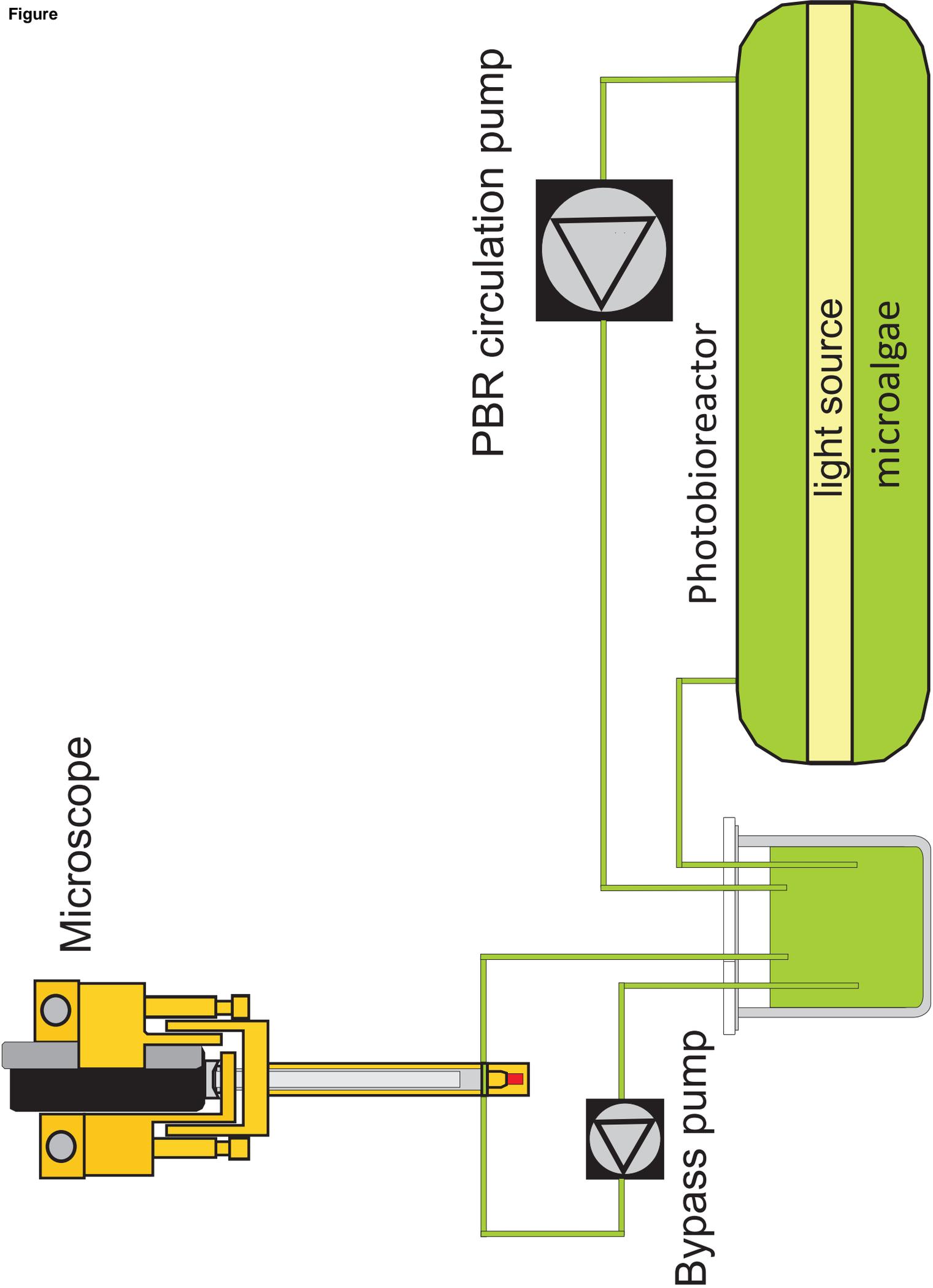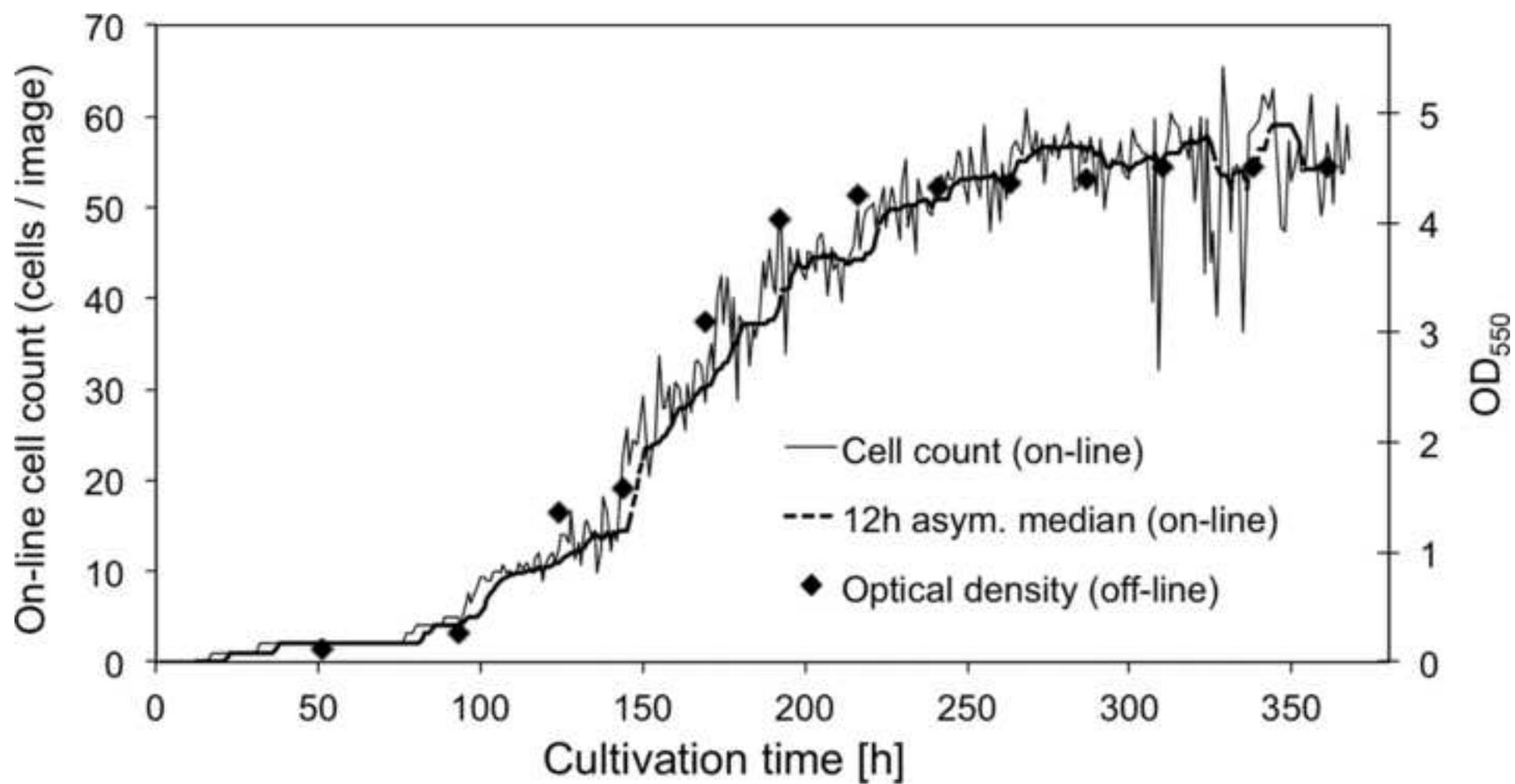
power supply

CCD - camera

linear stages

objective tube

tube
(forms measuring zone)

microscope body

condenser lens

light source

measuring zone
(sterile zone)

Figure

Microscope

PBR circulation pump

Photobioreactor

light source

microalgae

Bypass pump
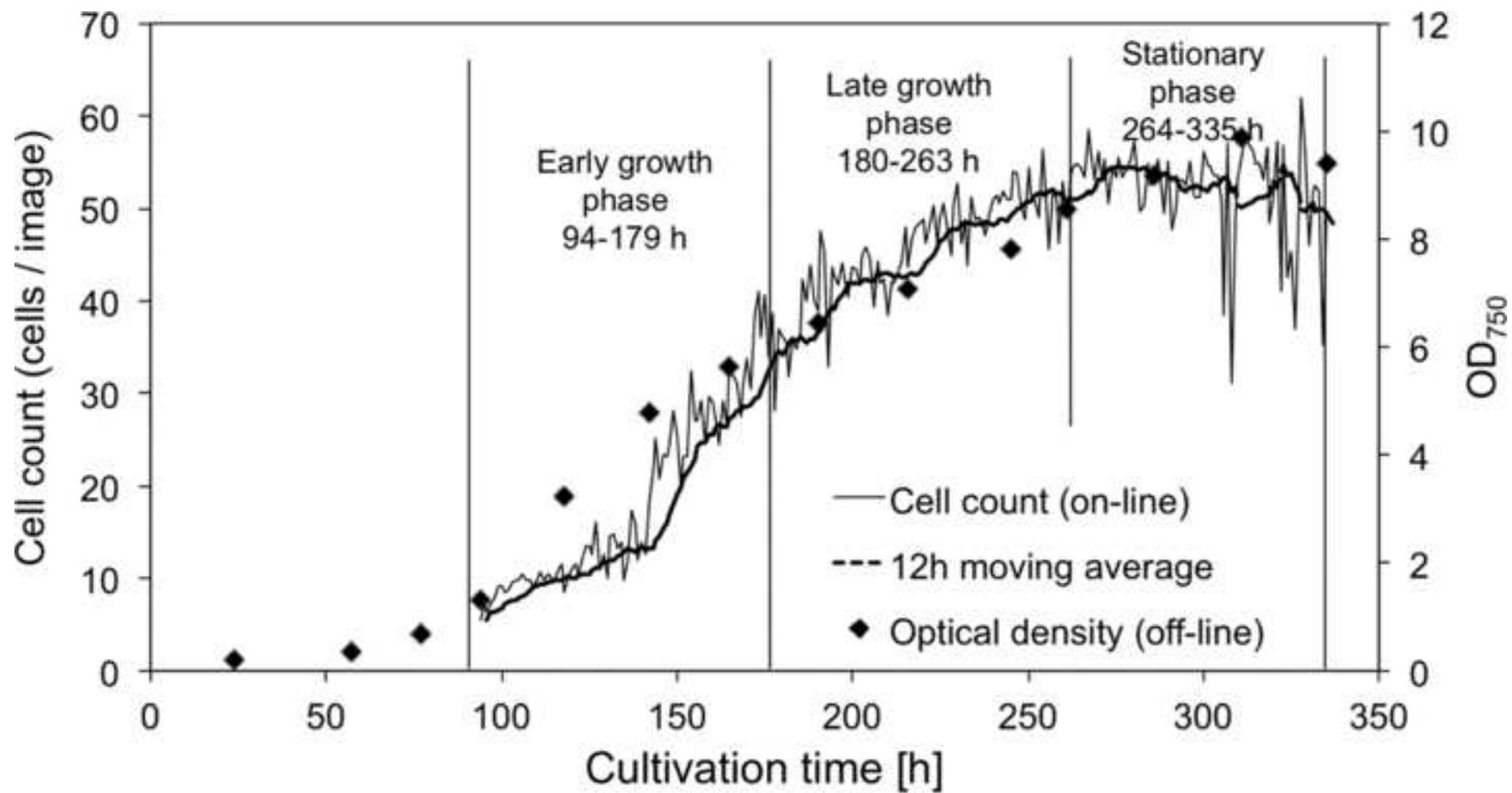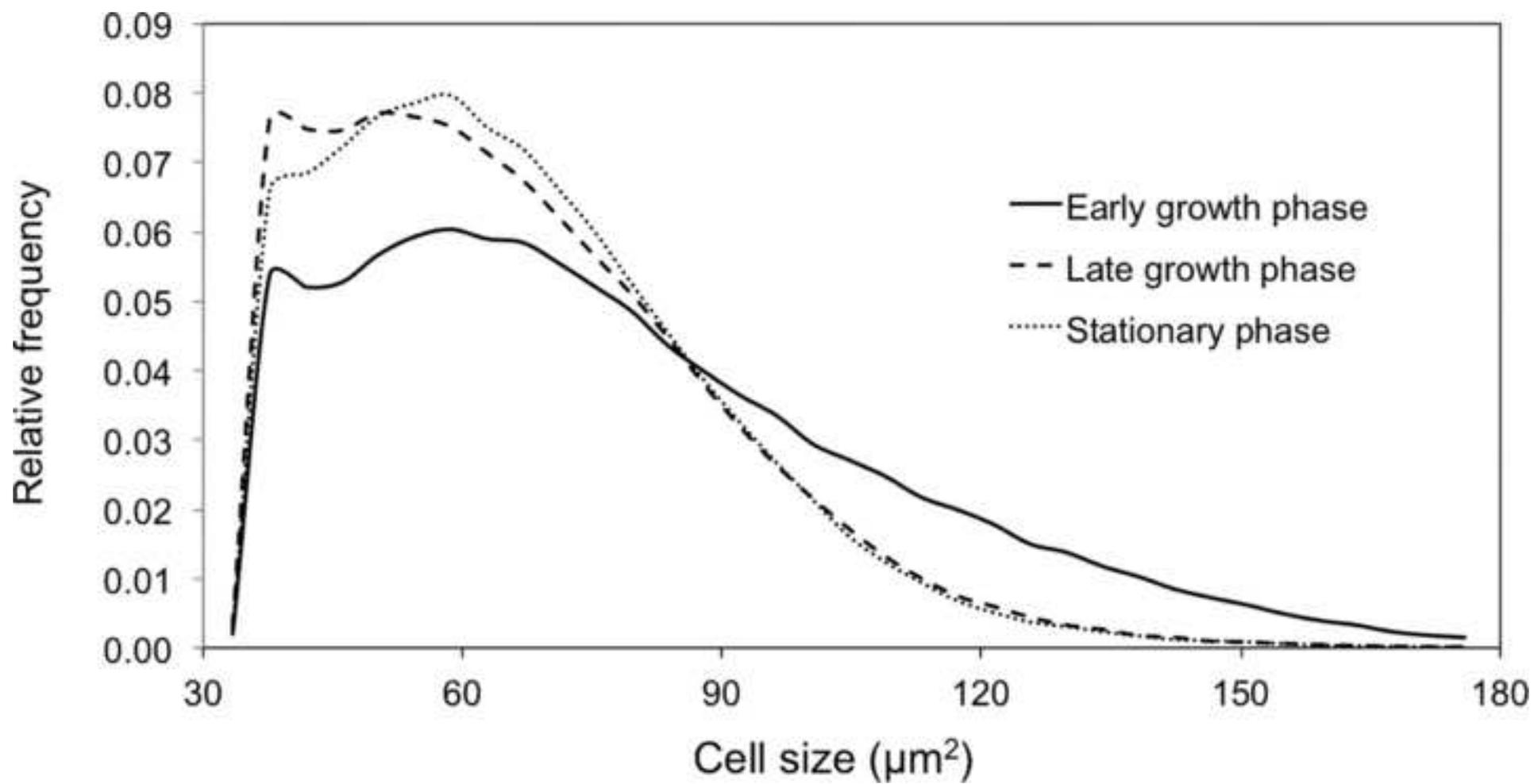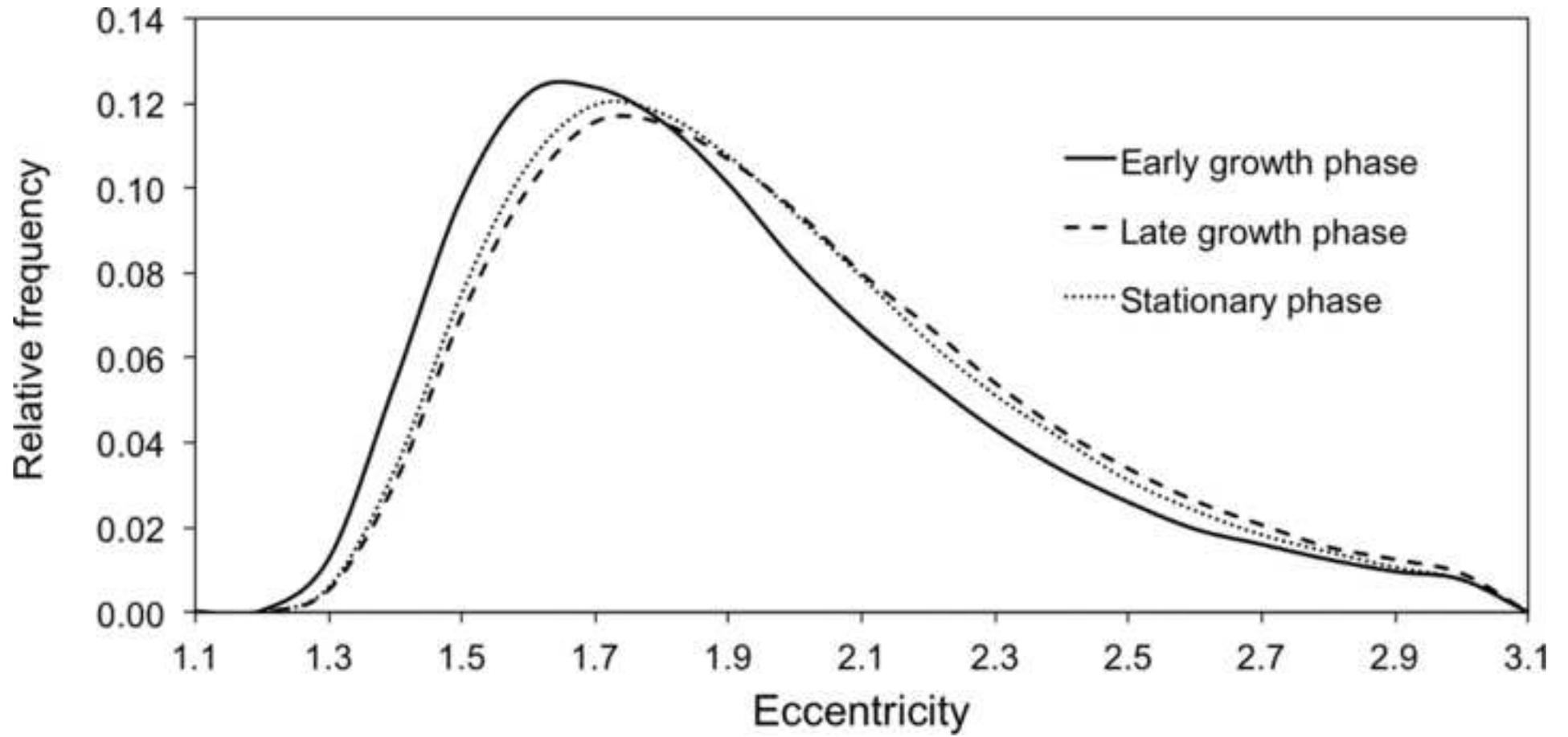
Supplemental Information for
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Figure S-1. Relative deviations of the ratio of off-line OD to on-line FTM cell counts from the mean of 0.83 computed over an entire cultivation of *Chlamydomonas reinhardtii*.
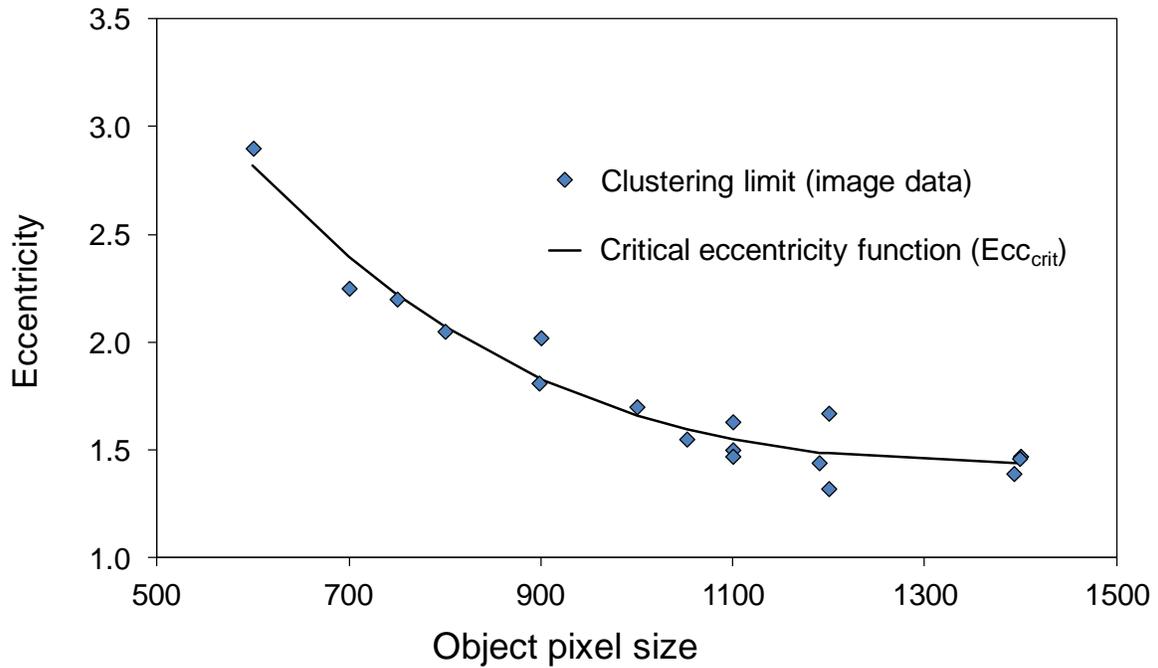
Figure S-2. Critical eccentricity function for analysis of *Chlorella vulgaris* image data. Manually selected image data (only in the early growth phase) was used to develop a critical eccentricity function for the size range 600 – 1400 pixels that serves to distinguish clusters and large single cells. All objects larger than 1400 px are assumed to be clusters, all objects smaller than 600 px are assumed to be single cells, and for objects in the size range 600 – 1400 px, a critical eccentricity is computed. All objects below the critical excentricity (provided by the function) for a given pixel size are assumed to be single cells. Objects classified as clusters can be then removed from further analysis concerning cell size and eccentricity.
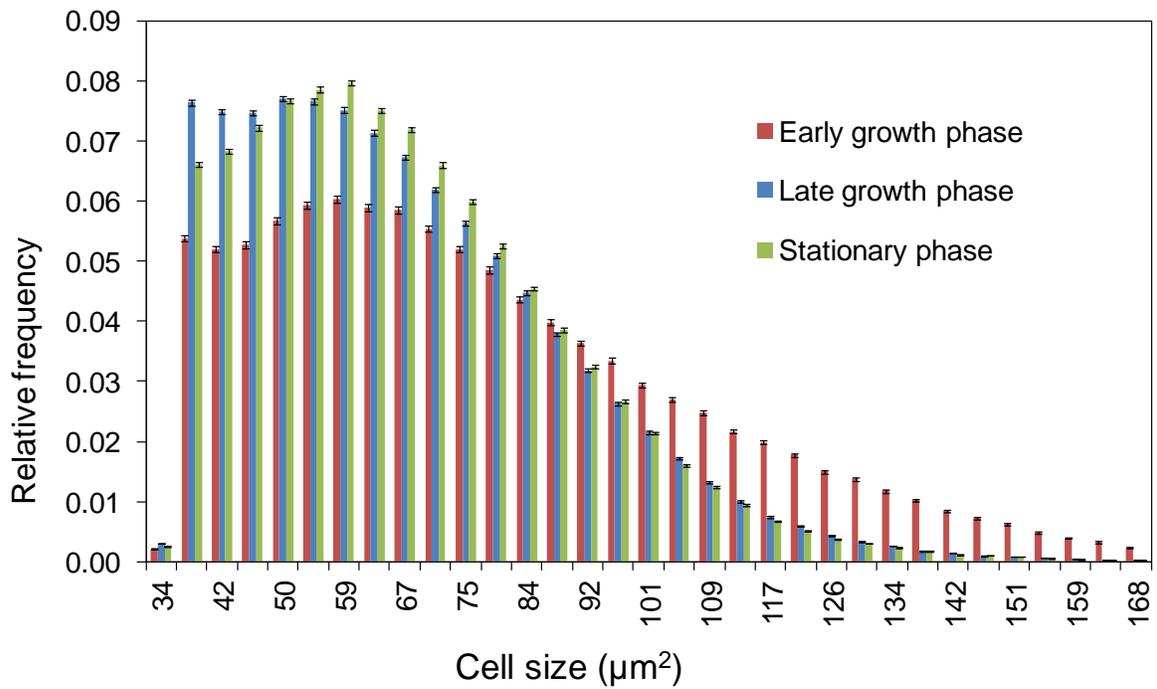
Figure S-3. Distributions of cell size during different phases of a *Chlorella vulgaris* cultivation in PBR-2 as measured by the FTM. The early growth phase (94-179 h), the late growth phase (180-263 h), and the stationary phase (264-335 h) are shown. Data resulting from cell clusters were removed using the experimentally derived function described in the text. Error bars were calculated for individual histogram bins by assuming Poisson distribution of count error; to normalize for relative frequency, the error in each bin with count N is equal to +/- $Sqrt(N)/Sum(N_1..N_{20})$.
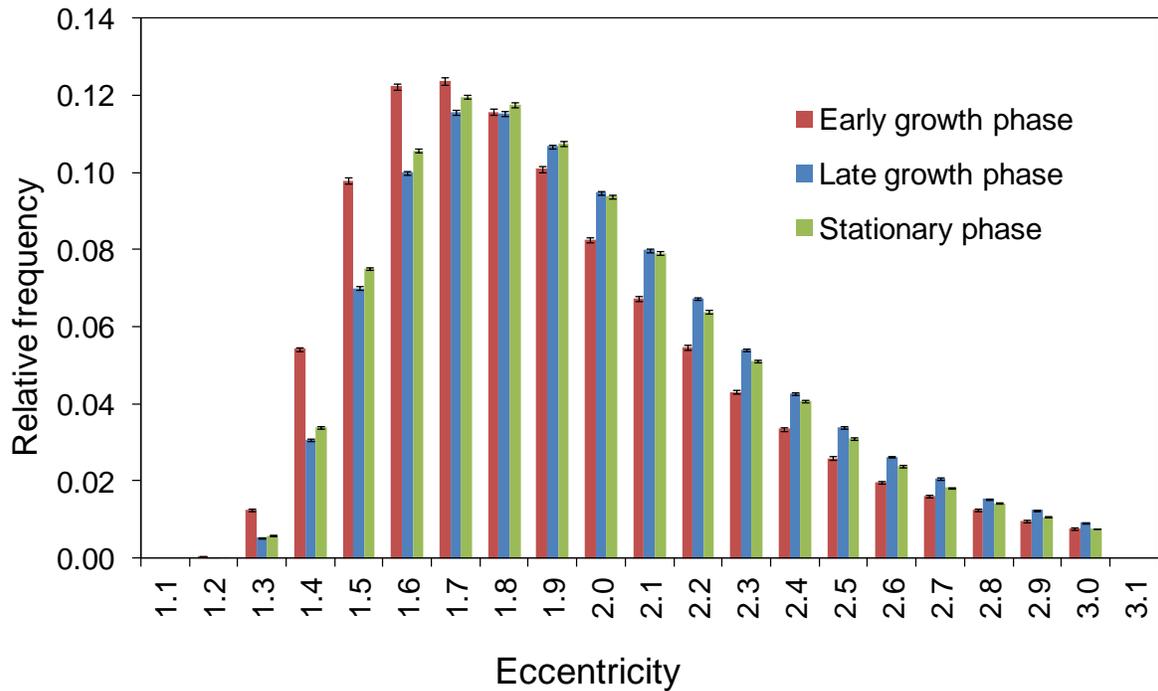
Figure S-4. Distributions of morphology in terms of eccentricity during different phases of a *Chlorella vulgaris* cultivation in PBR-2 as measured by the FTM. Eccentricity is defined such that a value of 1.0 corresponds to a circle and larger values are ellipses. The early growth phase (94-179 h), the late growth phase (180-263 h), and the stationary phase (264-335 h) are shown. Data resulting from cell clusters were removed using the experimentally derived function described in the text. Error bars were calculated for individual histogram bins by assuming Poisson distribution of count error; to normalize for relative frequency, the error in each bin with count N is equal to +/- $Sqrt(N)/Sum(N_1..N_{20})$.