Doctoral Thesis

Single-Cell Mass Spectrometry for High-Throughput Lipid Phenotyping of Chlamydomonas reinhardtii

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Single-cell mass spectrometry for high-throughput lipid phenotyping of *Chlamydomonas reinhardtii*

A thesis submitted to attain the degree of

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presented by

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4 Population Screening of *Chlamydomonas reinhardtii* with Single-Cell Resolution Using a High-throughput Micro Scale Sample Preparation for MALDI Mass-Spectrometry ..........................55

4.1 Introduction ...........................................................................57
4.2 Materials and Methods ..........................................................59
4.2.1 Chemicals ...........................................................................59
4.2.2 Cell Culture ........................................................................59
4.2.3 Characterization of *C. reinhardtii* strains .........................59
4.2.4 Microarrays ........................................................................59
4.2.5 Spotting the cells onto microarray slides ..............................60
4.2.6 Cell lysis and MALDI-matrix application ..........................60
4.2.7 MALDI-FT-ICR-MS ..............................................................60
4.2.8 MALDI-TOF-MS .................................................................61
4.2.9 Differentiating strains ......................................................61
4.2.10 Statistical analysis of mixed strains ..................................61
4.3 Results ...................................................................................63
4.3.1 Single-cell sample preparation of microbial cells ..................63
4.3.2 High-resolution MALDI-MS for peak identification ...........63
4.3.3 Characterization and identification of strains on the single-cell level .................................................................66
4.3.4 Deterioration of strain-specific phenotypes in multi-cell mass spectra .................................................................68
4.4 Discussion .............................................................................70

5 Single-cell MALDI tandem-mass spectrometry: unambiguous assignment of small biomolecules from single *Chlamydomonas reinhardtii* cells .........................................................72

5.1 Introduction ...........................................................................74
5.2 Experimental Section ............................................................76
5.2.1 Chemicals ...........................................................................76
5.2.2 MS/MS of lipid extracts .....................................................76
5.2.3 Sample Preparation for single-cell MS/MS .........................76
5.2.4 Single-cell MS/MS ...............................................................78
5.2.5 Data analysis ........................................................................78
5.3 Results and Discussion ..........................................................79
5.4 Conclusions .........................................................................84
6 Single-cell Mass Spectrometry Reveals the Importance of Genetic Diversity and Plasticity for Phenotypic Variation in Nitrogen Limited Chlamydomonas

6.1 Introduction

6.2 Materials and methods

6.2.1 Microbial strains and culture conditions

6.2.2 Nitrate limitation experiments

6.2.3 Single-cell mass spectrometry

6.2.4 Spectral processing of MS data

6.2.5 Data analysis for single cell mass spectrometry

6.3 Results

6.3.1 Single cell mass spectrometry

6.3.2 Analysis of variance within populations

6.3.3 Microscopy and population level measurements

6.4 Discussion

7 Conclusions and Outlook

7.1 Bioanalytical Questions

7.1.1 Transferability to other systems

7.1.2 Compound classes

7.1.3 In search of an “internal” standard

7.2 Biological Questions

7.2.1 Impact of natural selection

8 Appendix

8.1 Appendix to Chapter 4

8.1.1 Supplementary Tables

8.1.2 Supplementary Figures

8.2 Appendix to Chapter 6

8.2.1 Supplementary Tables

8.2.2 Supplementary Figures

8.2.3 Supplementary Material and Methods

9 References
Zusammenfassung

Abstract

Fueled by new biological insights the demand for new single-cell technologies has increased tremendously over the last decade. The knowledge gained based on these techniques benefits both fundamental and applied fields of biological and medical research. However, the transfer of most of these techniques to microbial systems is plagued with great difficulties. Moreover, still only very few techniques are available to characterize small molecules in single cells. The scope of this thesis was to develop a method based on matrix-assisted laser-desorption/ionization mass spectrometry to collect mass spectra of small molecules extracted from single cells. This allows direct access to the molecular composition of single cells. Previous work in this field had shown that matrix-assisted laser-desorption/ionization mass spectrometry provides the sensitivity to produce spectra from single cells. Since mass spectrometry is a destructive method, high-throughput is an absolute necessity to create meaningful biological insights, e.g. assessing cellular phenotypes in their population context. Thus the first and most crucial step of my thesis was to develop an analytically robust protocol to increase throughput and reproducibility. Furthermore we wanted to test if the method could provide only qualitative or even better quantitative information representative of the molecular composition of investigated cells. A protocol was developed for *Chlamydomonas reinhardtii*, a green freshwater microalga, which allows the detection of major lipid and pigment classes. In a second step we demonstrated the mass spectrometric power of the protocol in single-cell tandem mass spectrometry. Tandem mass spectrometry produces specific fragments from individual ions detected and therefore allows direct molecular assignment of signals detected in single cells. The third and most important step was to demonstrate the utility of the method to generate biologically relevant information. We used the method to follow different *C. reinhardtii* populations in a time series experiment over nine days. The experiment included a genetically diverse and two isoclonal lineages, which were
cultured under nitrogen deplete and replete conditions, to investigate the impact of genetic diversity and plasticity on the phenotypic variation in nitrogen limited cultures, as measured by differences in lipid and pigment composition. The analysis of almost 4000 single-cell spectra revealed that the genetically diverse culture displayed marked heterogeneity in the lipid composition throughout the experiment while the isoclonal lineages followed more homogeneous phenotypic trajectories. The genetically diverse population was also the only population which could maintain a fraction of cells seemingly unaffected by the nitrogen limitation in their lipid composition. Thus single cell mass spectrometry can generate insights into functionally relevant phenotypic variation and thereby contribute to the understanding of adaptation and phenotypic evolution.
Cells are the fundamental building block and the smallest viable unit in all life on earth [1]. The demand for studying biological systems with single-cell resolution is very high, both in fundamental and applied research [2-4]. This circumstance has boosted the development and improvement of many bioanalytical tools for single-cell applications [5]. This introductory chapter provides both an epistemological (1.1 Knowing single cells) and a technical perspective (1.2 Measuring the single cell) on current research the field of single-cell biology, with a special focus on the impact of single-cell technologies for the study of unicellular organisms.
1.1 Knowing single cells

Single-cell technologies supply biological, i.e. phenotypic or genotypic information from single cells. Being the basic unit of organization in many important biological systems it can yield information relevant for many different organizational levels and thereby cater to the needs of many different fields of biological science (Table 1).

1.1.1 A very special level of organization

In the elucidation of biochemical pathways and their regulation, the averaged response of a population can misrepresent the behavior of most of the cells within a population and thereby lead to wrong conclusions on the architecture of regulatory networks [2, 6]. Thus on the subcellular and the cellular level, single cell biology can render a clear insight on mechanisms and sources of cell-to-cell variation [6, 7]. In complex multicellular organisms these mechanisms include networks regulating cellular differentiation during development [4] or repair [8]. In unicellular organisms these include insights into regulatory networks which affect metabolic strategies [9, 10], reproduction or cellular behavior in different environments [11, 12]. The analysis of tissues and organs with single cell resolution can provide answers to basic questions on the extent and control of cell-to-cell differences and their functional impact on higher organizational levels. Cell-to-cell differences are expected to be highly controlled, e.g., in mammalian cells [13], however, there are also examples of pronounced cellular heterogeneity, such as the differential responses to drug treatments [14, 15] or in ageing [16]. Potential new insights therefore meet many needs from applied medicinal research, e.g., the evasions of regulatory control in cancer or the reprogramming of stem cells [4, 17]. Thus in applied research the understanding of biological systems with single cell resolution is of particular importance, whenever one or few cells can impact the fate of a larger biological entity.
In the context of this thesis the emphasis is laid on unicellular organisms i.e. the situation where each individual cell in a population is self-replicating and therefore a direct target for natural selection (Table 1). In this setting, single-cell technology can contribute to mechanistic and functional understanding of processes of ecological and evolutionary dimensions.

**Table 1** Simplified overview of different levels of organization in biological systems in complex multicellular organisms and unicellular organisms. Numbers indicate the general questions addressed by single cell technologies. The reproductive unit in each is highlighted in bold

<table>
<thead>
<tr>
<th>Level of Organization</th>
<th>Complex multicellular organisms</th>
<th>Unicellular organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomolecular networks¹</td>
<td>Single Cell¹,²</td>
<td>Biomolecular networks¹</td>
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<tr>
<td></td>
<td>Tissue²</td>
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<td><strong>Individuum / Organism</strong></td>
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</tr>
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Questions addressed by single-cell technologies

1. Molecular mechanisms underlying cell-to-cell heterogeneity and it’s regulation
2. Extent and functional role of heterogeneity

### 1.1.2 Single-cell biology of unicellular microorganisms

Since the emergence of the first life on planet earth, cells have greatly increased in complexity of their internal structure. Cells have socialized and specialized, e.g. by division of labor, the formation of
1.1 Knowing single cells

colonies and biofilms [18]. Thereby these systems have attained highly complex internal structures that provide stability and flexibility to many of our planet’s most fundamental ecosystem functions, such as nutrient cycles [19]. Single-cell resolution in the study of these systems is especially important, since in contrast to multicellular systems, where only specialized germ cells transmit genetic information to the next generation, unicellular organisms have retained their ability to reproduce. Therefore, each single cell in these systems is a target for natural selection and in theory has the potential to dominate the population.

Due to the ability to manipulate environments, the short generation time and the elaborate molecular toolboxes available, microorganisms further lend themselves well to study of experimental evolution [20, 21]. Experimental evolution is crucial for the generation of empirical evidence to many modelling approaches, which rationalize mechanistic aspects. The most impressive example in this area of research is the \textit{Escherichia coli} long-term evolution experiment run in the lab of Richard Lenski that runs since 1988 and currently spans about 60’000 generations [22]. The experiment has seen profound changes in basic metabolism such as the use of citrate as a primary carbon source, which the wild-type \textit{E. coli} cells were incapable of [23]. Additional examples of laboratory evolution include the evolution of multicellularity in yeast [24] or the adaptation to heterotrophic growth [25] or high salinity in \textit{Chlamydomonas reinhardtii} [26, 27].

Another aspect, which renders single-cell analysis particularly important to microbiology, is that an estimated fraction of less than 1% of currently described microbe species can be cultured in the lab [19]. The advances in the field single-cell analysis have recently given us a glimpse of this vast store of metabolic potential, that has so far eluded scientific study [28-30]. Advances in the study of genes and phenotypes can thereby provide access to important information for pharmaceutical, chemical and biological science.
1.1.3 Origin of cell-to-cell variation

Despite its importance, unraveling mechanistic origins of both genotypic and phenotypic variation in microbes is still in its infancy. Progress in microscopic tracking of individual cells has recently revealed that ageing [31] and the stochastic partitioning of cellular compartments is a common feature in cell division and reproduction even in seemingly symmetric cell division of bacterial cells [32, 33]. However, the universality of ageing and the importance of stochastic partitioning for cellular heterogeneity is still under debate [34]. Genetic diversity is an important source for phenotypic variation and an important constituent of biodiversity since it is highly correlated with the resilience and the productivity of biological systems [35], [36]. Genetic diversity is a population resource that can be greatly reduced by natural selection but is also generated and maintained by different molecular and behavioral mechanisms in unicellular organisms. Different regions of bacterial genomes, for example, show large discrepancies in terms of mutability, with ‘contingency’ genes that are highly mutable and so-called ‘housekeeping’ genes with low mutability [37]. What is more, environmental stress can, for example, induce enhanced mutation rates and competence in bacterial cells [38] while in eukaryotes stress can lead to a switch from asexual to sexual reproduction [39].

But even in the absence of genetic variation, that is in isogenic populations, there is plenty of evidence for non-genetic variation between cells [2, 3]. Non-genetic in this context does not mean that it is not heritable, but stands for phenotypic variation which cannot be accounted for by the presence of different genotypes contained within the population. Non-genetic variance between cells is often termed phenotypic plasticity or phenotypic heterogeneity [40]. The concept of phenotypic plasticity was inferred from observations in differential developments of complex multicellular organisms [41], e.g. plants adapting to low light conditions. However, the concept is also used in microbes when genetically identical cells respond with different reaction norms to environmental triggers, e.g. toxicity or nutrient...
limitation. Phenotypic heterogeneity is a more recent concept, which refers to the constitutive expression of differential phenotypes in a populations living in a seemingly homogeneous environment [40]. Phenotypic heterogeneity can be a symptom of a population strategy such as stochastic switching[42] or bet hedging [43].

The mechanistic origin of non-genetic variation among cells can be intrinsic noise, i.e. stochastic expression of genes [44], or extrinsic noise, e.g. the complex outputs of gene regulatory networks [6, 45]. In yeast, for example, the number of transcription factors for a certain gene was shown to be positively correlated with its phenotypic plasticity [46]. Differential transcription is a powerful tool for generating phenotypic variation. Genome wide screening of stochasticity in transcriptional promoters in *Escherichia coli* showed that expression of virulence genes was particularly noisy [47]. It is therefore likely that transcriptional noise constitutes a tool for generating phenotypic variance in traits that are likely to be a direct target for selection.

### 1.1.4 Functional role of cell-to-cell variation

Being the cause and consequence of evolution, variation is a fundamental feature of life and occurs at any given level of biological organization [21]. However the presence of different mechanisms for the generation of variation, that is genetic and non-genetic cell-to-cell variation, poses questions on the conditions that would favor their evolutionary emergence. Two examples of situations that could evoke non-genetic strategies are frequent occurrence of genetic bottlenecks [48] or an evolutionary history of unpredictable environmental changes, during which the severity and frequencies did not allow for timely adaptation [41].

Genetic bottlenecks can greatly reduce genetic diversity in a population, e.g., the infectious dose of some pathogens is close to a single individual [49]. In order to establish an infection and thereby source a new population, pathogens have to adapt to drastic environmental changes including evading the host immune responses.
Protozoan parasites usually have complex reproductive cycles including many different cellular states each of which shows marked heterogeneity [48]. For example *Plasmodium falciparum* merozoites reproduce asexually in their host’s blood but display marked differences in transcription [50].

Another situation which can lead to the emergence of non-genetic phenotypic variation are fluctuating environments that do not allow for adaptation [41]. In these situations phenotypic diversification of a isogenic population, such as in the stochastic switching, can lead to an higher long-term fitness of populations [51].
1.2 Measuring the single cell

This part of the introductory chapter gives an overview of current technologies that give access to information from single-cells within their biological context (see 1.2.1 Insight-driven single-cell measurements). Rather than give a comprehensive overview, special emphasis is laid on state-of-the-art analytical techniques that can be applied to unicellular organisms with high throughput.

1.2.1 Insight-driven single-cell measurements

An important aspect of single-cell biology that is often misunderstood in technological contexts is that a biological researcher is in most cases not interested at studying one cell only. This is very much in contrast to the analytical scientist who is challenged by the sensitivity of measuring molecules contained in a single cell [52, 53]. In fact, the biological information that can be obtained from a measurement taken on a single or a small number of cells is very limited [7].

There are two main reasons for this, the first is simply a statistical one; variation in and between cells is a statistical property and can thus only be measured by a sufficient sampling [7]. The other equally important reason is that to gain biological information, it is obligatory to study the single cell in its functional context [6, 40]. In a recent review, Wu and Altschuler argued that at close enough inspection every biological systems displays heterogeneity and that rather than simply measuring variation, biological research should concentrate on identifying biologically functional cell-to-cell variation [7]. To achieve this, it is necessary to study the single cells in their biological context that is as a part of a higher order assembly, e.g. a population, a tissue, an organ, an organism or an ecosystem. These aspects have important consequences for the analytical methods since they should supply either minimal invasiveness [4], thus allowing to follow a small number of cells over a longer time period, or high
throughput [7, 40]. High throughput implies that the methods can capture a representative cross section of cells in the system studied.

Analytical technologies, which are established in single-cell biology laboratories comply with these criteria, e.g. destructive techniques provide high throughput, such as single-cell genomic analysis, single-cell transcriptomics[54] or mass cytometry, while non-destructive technologies can be applied to a smaller number of cells in-vivo, such as in time-lapse microscopy [55].

1.2.2 Methods for single cell analysis

While the information on the genotype of a single cell can be obtained by sequencing tools, phenotypic information can be extracted by a variety of different methods. The principal method for investigating phenotypes in single cells is and has always been microscopy. This raises the questions why people take great efforts to develop other methods. There are various limitations of microscopic techniques many of which are due to limitations of the applied probes, e.g. fluorescent markers [56]. These major issues include (i) the limited parallelization possibilities due do overlapping excitation and emission bands [57], (ii) the in vivo applicability restricted by probe toxicity and cellular damage caused by the labelling process, (iii) the invasiveness and alterations in chemical behavior of biomolecules inflicted upon introduction of reporting fluorescent probes, as well as (iv) insufficient specificity for labeling for small molecules.

1.2.2.1 Single cell gene and genome sequencing

Sequencing of DNA has made great strides by reducing the cost, and increasing the throughput and the quality (lower sequencing error and increased read length) of sequencing information. For a general overview of traditional and current DNA sequencing technology see Figure 1. The first DNA molecule extracted from a single sperm cell was sequenced in the late 1980ies [58]. Despite the immense progress made in depth [59, 60] and throughput [61], single cell sequencing still produces data of significantly inferior quality compared to standard
bulk techniques [62]. Furthermore, there are various complications in the transfer of single-cell genome sequencing protocols from mammalian to microbial cells [62, 63]. Major obstacles include cell lysis, contamination and genome amplification.

Cell lysis efficiency is highly variable and was estimated to be around 40 % in microbial cells, but can be as low as 0% [64]. It must thus be assumed that there are significant sampling biases in current metagenomics datasets. Harsh extraction protocols are usually needed for cell lysis that can damage DNA and thereby interfere with extracted information content [63]. Issues in cell lysis and DNA extraction efficiency are aggravated by contamination. Especially when using degenerate primers such as in whole genome amplification (WGA) methods, every single DNA strand present in the sample is amplified.

Figure 1 Overview over previous (1st generation) and recent DNA sequencing technology (2nd generation). The third generation of sequencing technology is still being developed.
and the slightest contamination can easily swamp the targeted single-cell DNA [65]. Amplification itself is another challenge since errors affect the integrity of the sequence information [66]. Multiple displacement amplification (MDA) has recently replaced polymerase-chain reaction based methods for amplification of single-cell DNA since it results in both bigger fragments and lower error rates [67, 68].

The analysis of genomes reveals amazing new insights on uncultivated organisms [28] and has reached an information depth at the single cell level that is unmatched by other single-cell techniques. However from a single cell biology perspective, it would be highly desirable to further improve read length and fidelity to gain more independence from the consensus sequences of genome references [62, 64]. Furthermore, the compatibility of genomic sequencing to other phenotypic readouts is highly desirable in order to perform integrated studies on both genotype and phenotype of the same cell.

1.2.2.2 Single-cell transcription analysis

The analysis of RNA from single cells depends on reverse transcription to cDNA which is then sequenced. Transcriptomics, i.e. the highly multiplexed analysis of transcripts from single cells has recently made significant advances in throughput [69] and has even entered biological labs in the form of the commercially available Fluidigm C1 system [70]. The system provides a fully integrated platform which relies on the Illumina sequencing technology. However, initially developed for mammalian cells, the system has not yet been successfully transferred for the analysis of microbial samples. Again transferability to microbial cells is a major issue, mainly due to the lack of extraction techniques compatible with both the decomposition of microbial cell walls and the biomolecules employed in the transcriptomic workflow. Thus the analysis of the messenger RNA in microbes still relies on targeted microscopy techniques such as fluorescence in-situ hybridization (FISH) [71].
1.2.2.3 Single-cell protein analysis

Despite the great advances in the use of in-vivo fluorescent protein markers [72, 73] the demand for a more holistic studies on protein networks in single cells is great [74]. The many proteins in a cell represent a highly dynamic network in which proteins are being produced, regulated in their functional roles and degraded. These dynamics at least partly explain why protein abundance and activity often show very poor correlation [75]. Thus to understand the dynamics and accurately describe the activities of proteins single-cell resolution will play an important part. The main difficulty to overcome from the analytical side is the small amount of each individual protein present in a single cell. It has been estimated that a yeast cell weighing approximately 50 pg contains around 5 pg of total protein mass [76]. The few thousand different protein expressed in a single yeast cell show extreme variance in their abundance, from a few to a millions of copies [77]. However, as mentioned the abundance of a protein is not necessarily equivocal with its functionality, since post-translational modifications can greatly modify protein activity. Mass spectrometry, as the principal method for identification of proteins in biology [78], has taken several very early attempts at measuring proteins from single cells [52, 79, 80]. The proteins detected were, however, restricted to overly abundant proteins, such as hemoglobin in red blood cells [52]. Thus, the implementation of mass spectrometry for the detection of more biologically relevant proteins directly from single cells will require a significant boost in sensitivity.

A very elegant example which circumvents this sensitivity issue is a technique called mass cytometry [81]. It consists of a hyphenation of immune-labelling, flow-cytometry and inorganic mass spectrometry. The detection of proteins is based on labelling by antibodies which supply specificity. The antibodies in turn are labeled with rare-earth metal tags that are detected after ionization using an atomizing plasma torch by a time-of-flight mass spectrometer. To overcome sensitivity issues each antibody contains numerous chelated rare-earth metals. This method can therefore extend the number of proteins measured
from a single cell to forty [82]. The dependence on antibodies in the labelling efficiency, specificity and availability is a major restriction of the method even in mammalian cells. The application of the method is thus centered around the analysis of surface antigens, where offers impressive insights for immunology [83] and cancer research [84]. The immunohistochemistry involved severely limits the method in its applicability to microbes, where successful application has so far been restricted to the analysis of surface antigens [85].

1.2.2.4 Small molecule and isotope analysis in single cells.

There are a variety of analytical approaches that allow for the detection of small molecules and metabolites from single cells. Most of these approaches are still in the development stage due to issues in reproducibility, user-friendliness and throughput. The analysis of intact small biomolecules is mostly done by mass spectrometry approaches such as matrix-assisted-laser desorption/ionization mass spectrometry [86], surface-assisted laser desorption/ionization mass spectrometry [87], or electrospray ionization [88, 89]. A difficulty in using both MALDI or surface-assisted techniques such as nanostructures [87] is that the sample preparation, which can be difficult, is absolutely crucial for the success of the measurement. Live-cell mass spectrometry, a technique that relies on electrospray ionization, uses a sharp tip to extract cytosolic fluid from single cells which is then diluted and sprayed into a mass spectrometer for analysis [90].

MALDI mass spectrometry has a distinct advantages over ESI for the analysis of small molecules in single cells: (i) strong dilution of the sample is absent, since it relies on the ionization of molecules in the solid, i.e. crystallized, state [91], (ii) MALDI as an ionization method is much less susceptible to the high salinity of biological samples and (iii) the speed of spectral acquisition especially in combination with time-of-flight mass analyzers allows for the analysis of samples with high throughput (also see Chapter 2.2.3 Time-of-flight ion separation and detection).
The elemental analysis of single cells is more successful partly due to the fact that the high energy density employed during atomization of biological material yields high ionization efficiencies. Especially nanoscale secondary-ion mass spectrometry (NanoSIMS) has been successfully applied in the study of nitrogen fixation in single bacteria [92, 93]. However the information content is of course highly diminished by the destruction of molecular information.
2 Theoretical Background

This chapter supplies theoretical background on both the main organism investigated and the mass spectrometric technique that was optimized to develop a protocol for the mass spectrometric phenotyping of populations with single-cell resolution. Rather than a comprehensive overview this chapter provides the necessary basic knowledge for understanding the rationale of the developed method for single-cell analysis.
2.1 The model: Chlamydomonas reinhardtii

*Chlamydomonas reinhardtii* is a green freshwater microalga. Belonging to the order of Volvocales, *C. reinhardtii* is placed within a phylum including different stages in the transition between unicellular and multicellular colonial alga, e.g. the genus Volvox [94]. *C. reinhardtii* itself is unicellular and biflagellate but can form palmelloids as a response to environmental triggers like iron toxicity or predation [95]. The lifestyle of *C. reinhardtii* is highly flexible and can switch between photo- and heterotrophy (chemical energy can be generated from oxygenic photosynthesis and assimilated from external carbon sources such as acetate) as well as between sexual (mating types + and -) and asexual reproduction [96]. In the lab *C. reinhardtii* is usually grown asexually by culturing mating types isolated from each other.

2.1.1 Cellular architecture

The cell architecture of *C. reinhardtii* is shown in Figure 2. Cell size ranges from 7-10 µm. Major structures include the flagella, the cell wall, the chloroplast, the pyrenoid and the nucleus [97]. The cell wall is the outer barrier and the mechanical and chemical safeguard. It consists of seven layers of hydroxyproline-rich glycoproteins that form a highly cross-linked molecular meshwork [98]. In contrast to plant cell walls, the *C. reinhardtii* cell wall is devoid of cellulose [97], which is a common target for lytic enzymes in plant sciences. The chloroplast is the organelle that hosts the photosynthetic protein complexes within the thylakoid membrane [97]. Photosynthetic processes are well studied in *C. reinhardtii*, since they are very similar in land plants, which are evolutionary derived from green alga. Chlorophyll a and b are the photosynthetic pigments absorbing light in photosystems and antenna proteins. The pyrenoid is a globular structure that can be distinguished under the microscope [99]. It consist of a specialized region of the chloroplast where carbon dioxide fixation takes place. Further structures include mitochondria, starch grains and lipid bodies the latter two serving as storage compartments.
Figure 2 Basic features of *C. reinhardtii* cell architecture. A schematic representation of the cell is shown in A and a thin-section TEM of a cell in B. Photosynthesis takes place in the vast thylakoid membranes of the chloroplast which contains its own DNA. The pyrenoid is a specialized region where carbon fixation occurs which shows in B as the starch granules forming round it. Two flagella provide motility and a strong glycoprotein cell wall robustness and resistance towards osmotic stress. (TEM image in the public domain kindly provided by the Dartmouth Electron Microscopy Facility, Dartmouth College, UK, http://remf.dartmouth.edu/imagesindex.html)
2.1.2 Lipid composition and metabolism

Lipids provide essential cell functionalities such as cell compartmentalization by forming membranes or embedding proteins that are responsible for major cell functions. However lipids can also store chemical energy at very high density. Since both mitochondria and chloroplasts contain genomic information of their own some lipids are directly synthesized on site, e.g. thylakoid lipids [100]. Biosynthesis of other extraplastidic membrane lipids are encoded in the nuclear genome. Based on this ontological distinction there are plastidic and extraplastidic lipids [100]. All major lipid classes present in *C. reinhardtii* are depicted in Figure 3. In lipid composition *C. reinhardtii* shows many similarities with higher plants but also some clear distinctions. For instance phosphatidylcholine is absent and functionally replaced by diglyceryl-trimethyl-homoserine (DGTS) that are a major constituent of extra-plastidic membranes [100]. Other extra-plastidic lipid classes include phosphatidyl-inositols (PI) and phosphatidyl-ethanolamines (PE). Major lipids in the thylakoid membranes are glycolipids such as mono-galactosyl-diacylglycerols (MGDG), di-galactosyl-diacylglycerols (DGDG), sulfoquinovosyl-diacylglycerols (SQDG) and phosphoglycerolipids (PG). Triacylglycerols (TAG) are storage lipids that accumulate in lipid droplets within the cytosol. Lipid droplets are known to be formed as a stress response to various stresses such as high salt content, nitrogen, phosphorus or iron limitation [101, 102].
Figure 3 Chemical structures of the most abundant lipid classes found in *C. reinhardtii*. Notably DGTS replaces phosphatidylcholine.
2.1 The model: Chlamydomonas reinhardtii

2.1.3 Nitrogen uptake and metabolism

*C. reinhardtii* grows on different inorganic nitrogen sources including ammonium (NH$_4^+$), nitrite (NO$_2^-$) and nitrate (NO$_3^-$) [103]. Ammonium is preferred and will be consumed preferentially given the choice. This is mainly due to the fact that nitrate has to be reduced to ammonia, an energetically costly process, to be incorporated into biomolecules (see Figure 4). Nitrate is imported into cells by transporters (NRT1 and NRT2) and is reduced to nitrite by a cytosolic enzyme called nitrate reductase (NIT1 and NIT2). Nitrite is then imported into the chloroplast where it is reduced to ammonium by an enzyme called nitrite reductase (NIR). Both processes require significant amounts of reduction energy that is in provided in the form of NADH and ferredoxins in *C. reinhardtii*.

![Diagram of nitrogen uptake and conversion in C. reinhardtii](image)

*Figure 4* Overview of *C. reinhardtii* inorganic nitrogen uptake and conversion. Ammonium is the preferred nitrogen source as nitrate has to be converted to be reduced to ammonia in multiple energetically costly steps.
2.1.4 Nitrogen limitation

Nitrogen is a macronutrient often limited or fluctuating in natural environments. When limited in nitrogen, vegetative cells of *C. reinhardtii* initiate gametogenesis and start to accumulate starch and TAG’s [96, 104]. The gametogenesis results in the formation of gametes from vegetative cells and is probably triggered at a certain minimal ammonium concentration. Gametes can undergo cell division but are sexually competent in contrast to vegetative cells. It is therefore likely that due to low availability of ammonia in natural habitats *C. reinhardtii* cells are mostly in the gamete state [96, 105].

The accumulation of starch and TAG lipids has caught the attention of researchers interested in using algae for industrial biofuel production [101]. Accumulation of starch and TAG most likely alleviates reducing pressure of the excess light energy caught by the photosynthetic reaction center when cells stop growing but are still photosynthetically active [106, 107]. Both processes accompany the down-regulation of photosynthesis. Chlorophyll is not actively degraded, but its biosynthesis is stopped [108]. The nitrogen pool in cells is maintained by degradation of proteins and fast recycling of nitrogen in the form of amino acids, polyamines or nucleobases [109].
Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption-ionization is a physico-chemical process, which results in the formation of charged molecules, i.e. ions, in the gas phase, which can be transferred to and measured by a mass spectrometer. Together with electrospray ionization (ESI), MALDI was one of the two first “soft” ionization techniques that would allow the ionization of large intact biomolecules. In 2002 Koichi Tanaka (MALDI) and John B. Fenn (ESI) got awarded with the Nobel Prize in Chemistry “for their development of soft desorption ionization methods for mass spectrometric analyses of biological macromolecules” [110]. Since then mass spectrometry has made great strides with respect to detection limits and mass resolution [111].

For MALDI-MS the molecules to be analyzed (analytes) are usually mixed with a small UV-absorbing organic molecule - the MALDI matrix. In this mixture the MALDI matrix is present in great excess (usually about $10^3$-$10^4$ matrix molecules for every analyte molecule). The mixture is then co-crystallized by evaporation of the solvent before the sample is transferred into the vacuum of the MS. Since the MALDI matrix is highly absorbing in the UV range of the optical spectrum, it serves as mediator for transferring the energy absorbed during laser irradiation onto the analyte molecules. The main applications of MALDI-MS nowadays are polymer analytics, bio-typing of microbes [112] and imaging of tissue slices.

### 2.2.1 The MALDI process

For simplification, the MALDI process can be divided into three stages [113]:

1. Absorption of the laser radiation by the MALDI matrix
2. Ablation of the matrix/analyte co-crystals
3. Ionization of the analyte molecules
The discrimination between and the role of each individual process in ionization of the analyte molecules is still under investigation. The importance in understanding these processes is not just interesting from a fundamental physicochemical standpoint but also of high importance for the exploration of new matrices providing lower limits of detection and specificity for different compound classes [114] (see also Chapter 7.1.2 Compound classes).

The MALDI process starts with the irradiation of the sample with laser light. Nowadays pulsed UV lasers with pulse width in the nanosecond range are most widely used. There are two major types of lasers employed in commercial MALDI, the N\textsubscript{2} gas laser (emission wavelength 337 nm) and the solid state Nd:YAG laser (frequency tripled, emission wavelength of 355 nm) [115]. Commonly used MALDI matrices for the analysis of positive analyte ions include 2,5-dihydroxybenzoic acid, cinnamic acid or sinapinic acid [116, 117]. All of them show a distinct absorbance peak around 350 nm. Energy absorbed by the crystals leads to an ablation (also termed “desorption”) of the co-crystals [118]. The ablated material expands into the vacuum within the MALDI ion source and ions that are generated in this plume of material of are accelerated away from the sample target into the mass analyzer by an applied potential (see Figure 5A/B).

### 2.2.2 Ion formation in biological MALDI

The ion formation process in MALDI is still a matter of debate and there are probably multiple different mechanisms that contribute [114, 119]. MALDI mass spectra are characterized by predominately singly charged ions, which facilitates spectral interpretation compared to ESI mass spectra. Generally both negative and positive ions are formed in the ablation/ionization process. The ions formed comprise protonated / de-protonated analytes, radical ions as well as metal ion adducts. There are two basic mechanisms proposed for ionization in the positive ion mode:

In the photoionization model[120] the analyte molecules are assumed to be neutral in the matrix/analyte co-crystal. Upon laser
irradiation the matrix molecules (M) are charged via photoionization and can transfer their charge to the analyte molecules (A) in the expanding plume (see Equation 1 below) [121, 122]. This model can account for protonation of analytes such as peptides and amino acids since this protonation is numerically supported by the gas-phase acidity of some matrices[123].

\[
M + A \stackrel{h\nu}{\rightarrow} M^* + A \rightarrow [M-H]^− + [A+H]^+ \quad (1)
\]

Criticism on this model arises due to the fact that the energy of a single photon (3.3 eV for the frequency tripled Nd:YAG laser emitting at 355 nm) is not sufficient to ionize matrix-molecules in crystals and two-photon processes are much less probable. The formation of radical cations however is a strong indication of photoionization and thermal effects, a lowered ionization potential in the co-crystal as well as energy pooling from multiple photons could still account for photoionization [114]. The “lucky-survivor” model assumes that analyte molecules carry their charge already inside the matrix crystal, for instance by maintaining parts of their solvation shell. Upon ablation of matrix crystals clusters are formed some of which contain analytes that have retained their charge [124]. Many of them are neutralized in the expanding plume however the molecules detected by mass spectrometry are the “lucky survivors” of this process [125]. Investigations using pH-indicator molecules show that certain molecules can retain their charge inside the matrix/analyte co-crystal [126]. The formation of singly charged ions during MALDI from peptides and even proteins that accumulate multiple charges in solution is still a conundrum and can be rationalized relatively well by the lucky-survivor model. Thus a multiply charged protein (multiply protonated here for simplicity) is neutralized by deprotonated matrix molecule to a degree where just one charge remains on the luckily surviving protein ion:

\[
(n-1)M^- + [A+nH]^{n+} \rightarrow (n-1)[M+H] + [A+H]^+ \quad (2)
\]
Another important part of biological mass spectrometry, especially for the analysis of lipids is cationization with metal ions. In biological samples these include mostly Na\(^+\) and K\(^+\) ions which are omnipresent in biological samples. The attachment of these ions onto neutral analyte molecules most likely occurs in the MALDI plume and depends on the relative concentrations of analyte and cation in the plume and the residence time of the two in the plume[127]. The latter can be manipulated by the delayed extraction time parameter of MALDI mass spectrometers [128].

2.2.3 Time-of-flight ion separation and detection

Mass spectrometers are usually composed of three functional parts, the ion source, the mass analyzer and the detector [129]. The ion source is the part of the instrument where ions are generated. In time-of-flight analyzers this region is often under vacuum (10\(^{-7}\)-10\(^{-8}\) bar). The samples are introduced into the source on specialized sample targets. A good sample target for MALDI-TOF has to be electrically conductive and flat. Conductivity of the plate prevents a charge build-up on the target that impairs ion yield. More importantly the equipotential lines accelerating the ions will be more homogeneous on a high quality conductive target and therefore improve spectral quality (see Figure 5). As described previously the sample is ablated and the analyte ionized upon laser ablation. After ablation the plume of ablated material expands in the ion source (Figure 5A). Before acceleration of the ions into the mass analyzer the ions in the plume can expand and react for a fixed amount of time. This timeframe is set as a parameter called delayed extraction time and serves both in optimizing ionization conditions as well as enhancing mass resolution.

The physical principle of the time-of-flight mass separation is very simple: applying an electrical potential \(U\) accelerates the ions generated upon ablation in the source. The potential energy of the ions can be described as:

\[
E = zeU
\]
Where $z$ is the charge of the ion and $e$ the elementary charge. The ions are accelerated and enter a field-free drift tube. The kinetic energy of the ions, which can be described as:

$$E_{kin} = \frac{1}{2}mv^2$$

Thus ions travel at different velocities $v$ based on their mass-to-charge ratio ($m/z$).

$$v = \sqrt{\frac{2E_{kin}}{m}} = \sqrt{\frac{2zeU}{m}}$$

An oscilloscope measures the time the ions need from their initial acceleration until they reach the microchannel plate (MCP) detector (Figure 5B). Upon impact of an ion the MCP detector produces multiple electrons from secondary emission. There are multiple features in a modern MALDI time-of-flight mass spectrometer that enhance spectral parameters. One important aspect arises from the fact that upon ablation the initial kinetic energy of the ions differ resulting in differing time-of-flights and impaired peak resolution in the resulting mass spectra. This variance is reduced by two instrumental features.

First there is a time gap between the ablation and the onset of the accelerating potential called the delayed extraction time. Since ions with high kinetic energy upon ablation will travel a larger distance away from the target the acceleration potential will be lower, which will focus ions of the same mass. A second instrumental feature for refocusing the ion packets is the reflectron [130]. It works as an ion mirror and consists of a series of ring electrodes that create an electrical field gradient slowing the ions down and reflecting them (Figure 5C). Ions with the same mass but different velocities will thus be refocused due to different penetration depths into the reflectron. Both processes contribute substantially to the resolution of MALDI-TOF mass spectrometers, which can be as high a 30’000. Despite the fact that many more sophisticated mass analyzers can nowadays be coupled with MALDI sources only time-of-flight detection can keep up with the speed of laser ablation and thus take full advantage of the sample throughput that MALDI provides.
Figure 5 Graphical abstract of a MALDI sample ablated in the MALDI source (A) and the two main instrumental configurations employed in MALDI-TOF-MS (B, C). The principle of linear MALDI-TOF-MS is shown in B and reflectron MALDI-TOF in C.
2.2.4 MALDI imaging

High-throughput in acquisition has led to the application of MALDI as an imaging technique. Thereby a tissue [131] or a microbial colony [132, 133] is scanned with the laser and a spectrum collected from each position. Thereby a map of mass spectra is created that holds spectral information within spatial context. Recent years have seen significant advances due to progress both in sample preparation protocols [134] and instrumentation [135]. Sample preparation tools focus on lowering cross-contamination between cells during matrix application while progress in instrumentation mainly focused on ablation and ionization. MALDI laser beams can be focused down to the range of a few micrometers which combined with appropriate matrix application can render a resolution of 5-10 μm in tissue imaging [136]. Due to limited penetration depth of UV lasers the amount of material ablated decreases drastically with decreasing beam diameter challenging the sensitivity of the MS. Recently, post-ionization of ablated molecules using an additional wavelength-tunable laser on the ablated MALDI-plume was shown to increase sensitivity by two orders of magnitude [135]. However in microbial samples there is major trade-off between cross-contamination between adjacent cells and extraction efficiency when cells are in close proximity and since many prokaryotic cells are in the range of 0.5-1 μm it will still take significant progress until MALDI imaging will render single-cell resolution in bacterial colonies and biofilms.
This chapter reports a detailed and narrated protocol, a so-called standard operating procedure (SOP), of the workflow developed for single cell analysis in *C. reinhardtii* (see Chapter 4). It should assist everyone who tries to adapt or apply the developed protocol in their own research setting.

*This Chapter is adapted from:*
Abstract

Mass spectrometry based metabolomics is the highly multiplexed, label-free analysis of small molecules such as metabolites or lipids in biological systems and thus one of the most direct ways to characterize phenotypes. However, the phenotyping of populations with single-cell resolution is a great challenge due to the small number of molecules contained in an individual cell. Here we describe a microarray-based sample preparation workflow for MALDI mass spectrometry that has single-cell sensitivity and allows high-throughput analysis of lipids and pigments in single algae cells. The microarray targets receive individual cells in 1430 separate spots that allow the cells to be lysed individually without cross contamination. Using positive ion mode and 2, 5-dihydroxybenzoic acid as the MALDI matrix, the mass spectra unveil information about the relative composition of more than 20 different lipids/pigments in each individual cell within the population. Thus the method allows the analysis of cellular phenotypes in a population on a completely new level.
3.1 Introduction

Mass spectrometry (MS) is a pivotal tool for the analysis of metabolites in biological systems. Depending on the efforts taken, MS-based approaches can provide high sample throughput, highly multiplexed readouts and/or exact quantitation of metabolites. The main requirements for the development of a successful single-cell mass spectrometry method are high throughput, high sensitivity and selectivity with respect to a very complex biological matrix [137].

The main ionization methods employed in the analysis of intact biomolecules are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). MALDI is a soft ionization technique [121, 138], that allows the ionization and detection of intact biomolecules. It relies on the absorption of laser light and the transfer of charges by auxiliary matrix molecules. This still not fully elucidated ionization process results almost exclusively in the formation of singly charged ions greatly, which facilitates spectral interpretation [114, 139]. Most commercial instruments employ a UV-laser and a time-of-flight mass analyzer and feature very short spectral acquisitions times. Therefore, they permit to collect spectra in high-throughput fashion and allow spectral mapping of tissues or even whole organisms with high spatial resolution [140]. In selected examples specialized laser optics and careful sample preparation allows the imaging of tissue slices with single-cell resolution [141]. Therefore, MALDI is sensitive enough to provide spectra collected from the analytes contained in a single cell [91].

However, the analysis of single cells from liquid cell cultures of by MALDI-MS is struggling with the reproducibility in sample preparation and the lysis of microbial cells. This is why most attempts at using MALDI for analyzing single cells show very limited throughput.

The workflow described here employs instrumentation that is widely used in micro-arraying technology, namely a non-contact piezo-actuator driven sub-nanoliter dispenser [142]. This technology imparts high reproducibility and accuracy to all the steps of sample preparation.
Furthermore, we combined this with a dedicated MALDI target slide that provides 1430 distinct spots where individual cells can be placed for further processing and extraction (see Figure 6). First the spots are covered by a layer of MALDI matrix (DHB). Then the cells are applied to the spots on the target by spotting a cell suspension featuring an optimized cell concentration aimed at yielding the maximal number of single-cell events. Since the cells emit fluorescence upon irradiation with specific laser wavelengths, their fluorescence can easily be detected on the metal MALDI target slides. Then their metabolism is quenched and mechanical damage inflicted by fast-freezing the cells in liquid nitrogen. Only then the matrix dissolved in organic solvents is applied to extract lipophilic analytes and co-crystallize them with co-precipitating MALDI matrix during evaporation of the solvents.

In this fashion, this protocol for MALDI-MS can avoid the heavy dilution that usually hampers ESI analyses. The protocol was optimized using *Chlamydomonas reinhardtii* populations grown in liquid cultures. *C. reinhardtii* is a single-celled eukaryote and a regularly used model organism for the study of photosynthesis [143] and more recently for algal biofuel production [144]. Its highly robust glycoprotein cell wall is a good surrogate for microbial systems [145]. Therefore, the protocol can potentially be adapted to other microbial systems that show auto-fluorescence. Using 2,5-dihydroxybenzoic-acid as a matrix [146] in the positive ion mode, the method gives access to approximately 20 different lipids and pigments in *C. reinhardtii*. The identity of compounds was confirmed using FT-ICR mass spectrometry taking advantage of the very high mass accuracy and of CID based MS/MS measurements that were carried out at the population level. From the 1430 spots on the target slide, ideally around two thirds will be occupied by single cells. Thus one measurement can yield around 1000 single cell spectra per chip. The highly reproducible spectra are a highly interesting target for statistical analyses with respect to the detected signals as well as their relative abundance. This allows for categorization and characterization of different phenotypes within the population.
Figure 6. Schematic of the array. Spots on the edge (A1, A109, AY1, AY109) can optionally serve for spotter alignment. A All the wells are covered with a layer of matrix (10 drops of the DHB solution). B After the optimal number of drops is defined the whole array is covered with cell suspension resulting in many single cell covered wells. C Analytes are extracted from cells by recurrent application of MALDI matrix dissolved in acetone/water.
3.2 Materials

3.2.1 MALDI target slides

3.2.1.1 Chemicals/ Materials

1. Stainless steel plate (X5CrNi18 10, 1.25 mm thickness)
2. Acetone, HPLC quality (Sigma Aldrich, Chromasolv)
3. Sonication bath (Bandelin, Sonorex)
4. Non-fuzzing wipes
5. Glass jar for sonication
6. Water, for chromatography (Merck, LiChromasolv)
7. Chloroform (Sigma Aldrich, ReagentPlus)
8. Isopropanol, HPLC quality (Sigma-Aldrich, Chromasolv)
9. 50ml plastic tubes with screw caps, polypropylene

3.2.2 Single-cell sample preparation

3.2.2.1 Chemicals/ materials

1. Fluorescence dye 5μM in 3xSSC buffer for spotter alignment (e.g. CY3)
2. MALDI-matrix solution (10mg/ml 2,5-dihydroxybenzoic acid (Sigma Aldrich, 85707) in 90% acetone (Sigma Aldrich, Chromasolv) and 10% water (Merck, LiChromasolv), freshly prepared
3. Chlamydomonas reinhardtii cells in the form of liquid suspension cell cultures (see Note 1)
4. 1,5 ml Safe-lock, tapered-bottom vial, polypropylene
5. 384-micro well tapered-bottom microplate, polypropylene
Chapter 3

6. Liquid nitrogen
7. Long tweezers

2.2.2.2 Instrumentation
1. Confocal fluorescence scanner (Tecan LS 400), see Note 2
2. Non-contact microarray spotter (non-contact piezo-actuator driven spotting device e.g. Scienion S11) see Note 3
3. Centrifuge for 1,5 ml tubes (5415R, Eppendorf)
4. Desiccator connected to a vacuum/ nitrogen line

3.2.3 MALDI-MS analysis of single cells

3.2.3.1 Chemicals/ materials
1. Chlorophyll analytical standard (Sigma Aldrich, C5753)
2. Freshly prepared MALDI-matrix solution (10mg/ml 2,5-dihydroxybenzoic acid (Sigma Aldrich, 85707) in 90% acetone (Sigma Aldrich, Chromasolv) and 10% water (Merck, LiChromasolv)

3.2.3.2 Instrumentation
1. Mass spectrometer equipped with a UV-MALDI source (see Note 4)
Figure 7 Diagnosis for different problems that can occur during sample preparation process. Early cell lysis (1.) leads to a corrupted readout that no longer reports single cell properties. Inhomogeneous crystallization (2.) and incomplete cell lysis (3.) result in low signal response. In both cases the majority of the analytes are not ionized in the first case because the crystals are not ablated in the latter because the analytes are not being extracted.
3.3 Methods

3.3.1 MALDI target slides

3.3.1.1 Manufacturing Target Slides

1. MALDI targets are made from stainless steel slides (75mm x 25 mm) and cut with high precision (>100 μm) using laser from a big piece of stainless steel (30 x 30 cm) of 1.25 mm thickness using laser machining (Nd:YAG laser).

2. The slides are microstructured with the single-cell array spots using picosecond laser ablation. The array consists of 1430 spots (26 rows, 55 columns) with a diameter of 300 μm each and a 720 μm center-to-center distance. The spots showed a rough surface and were less than 1 μm deep. They serve as marks for orientation during sample preparation and MALDI-MS measurements and their rough surface facilitates the “focusing” of liquids and the crystallization of the matrix.

3. Slides should always be properly cleaned before use (see Note 5).

3.3.1.2 Cleaning MALDI target slides

1. MALDI target slides are scrubbed with an acetone drenched non-fuzzing wipe.

2. Slides are sonicated in a glass jar using solvents with different polarities. The following solvent conditions should be maintained for 30 minutes each in a sonication bath: 100% water, 100% isopropanol, 100% acetone, 100% chloroform.
3. After this cleaning procedure the slides are stored in 50ml falcon tubes in a nitrogen atmosphere.

### 3.3.2 Single-cell sample preparation

1. To check for potential contamination the stainless steel slides are scanned for chlorophyll fluorescence before the sample application (excitation at 630 nm, detection at 670 nm, detector gain: 120, 6 μm resolution). Scanning parameters are kept constant for the rest of the experiment.

2. The x-y stage of the piezo dispenser is aligned with the pre-structured array on the slide by spotting a fluorescent dye (e.g. CY3) into the corner spots on the slide (see Figure 6). Adjust positioning of the droplet spotter if necessary (see Note 6). Scan the alignment marks at 532 nm excitation and 570 nm detection.

3. A layer of matrix is spotted into each recipient spot by applying 10 drops of a 10mg/ml solution of DHB dissolved in 90% aqueous acetone.

4. To minimize the ion suppressing effect of the salts contained in the culture media during MALDI-MS measurements, cells are centrifuged at 2000 x g and re-suspended in deionized water. This procedure is repeated 3 times (see Note 7).

5. The cell number is adjusted to approximately $10^6$ cells per ml (1 cell per nanoliter, 0.5 cells per drop) using deionized water.

6. To determine the optimal number of drops for highest yield of single-cell events on the chip a dilution series is spotted. 3x3 spots are filled with 1, 2, 3, 5 and 10 drops of the cell
suspension.

7. The part of the slide that contains the dilution series is scanned and the number of drops that show the highest yield of single cells is determined (needed for step 8).

8. The cells are spotted using the previously defined number of drops into all the wells. A fluorescence scan is used to determine the number of cells in each spot. This information is essential to assign the mass spectra.

9. The slide is submerged in liquid nitrogen to quench the metabolism and to facilitate the analyte extraction (see Note 8).

10. The slide is returned to room temperature in a desiccator to prevent condensation.

11. The analytes are extracted by spotting the matrix dissolved in 90% aqueous acetone. 5 cycles of 10 drops are applied resulting in recurrent and more reproducible crystallization of the analytes and the matrix (see Note 9).

12. A fluorescence scan of the slide is performed after matrix application to check the lysis of the cells and the reproducibility of the extraction (see Figure 7).

13. The slide is stored at -80° C in a 50 ml falcon tube filled with nitrogen for MS measurements.
3.3.3 MALDI-MS

1. The target slides are reconstituted to room temperature just before the MALDI measurements in vacuum to avoid condensation of humidity.

2. The target slides are complemented with external chlorophyll standards premixed with MALDI matrix solution for external mass calibration.

3. The slides are loaded into the mass spectrometer and MALDI mass spectra are recorded in automated fashion for every spot using a spiral ablation pattern. The following conditions were successful on a AB Sciex 5800 MALDI TOF instrument: mass range 500-1000 m/z, 4300 a.u. laser energy, 400Hz pulse rate, detector voltage: 1.770 kV, 200 ns delayed extraction time, 12 shots/sub-spectrum, 50 sub-spectra. Measurement time was 2 h 45 min per slide, 6.5 sec per spot.
3.3.4 Data Analysis and Interpretation

1. The number of cells, as extracted from the fluorescence scan in step 8 (3.2 Step 8) should be attributed to each mass spectrum.
2. Peak lists containing the information on the m/z value, the peak area, the S/N value and the resolution of each signal with S/N > 10 are exported for all of the measured spots.
3. To check for cross contaminations and lysis efficiency the dataset should comply with the following criteria (more information see Note 9-12):
   a. Mass spectra of spots that lack auto-fluorescence must not show cell-derived signals, but show signals of DHB.
   b. More than 90 % of the spots occupied by a single cell should detect chlorophyll a.
4. Information extracted from the data includes the presence and absence of signals in the cells as well as the relative composition of the detected signal in the cells.
3.3 Methods

3.3.5 Notes

1. So far the protocol was used most successfully applied to *C. reinhardtii* cells grown in TRIS-acetate phosphate (TAP) medium. However, this protocol can potentially be optimized for any fluorescent microbe.

2. Slide scanning after cell spotting and lysis, respectively, is important to measure the number of cells present in each spot, and for optimizing the lysis protocol. Chlorophyll fluorescence, excited at 633 nm and detected at 670 nm, shows little background fluorescence by scattering or DHB auto-fluorescence. Thus, the presence of chlorophyll can be traced by fluorescence scanning at all stages of the experiment (including lysis and co-crystallization).

3. The spotter should offer highly reproducible, customizable, non-contact spotting of drops in the sub-nanoliter-range. For our experiments we used an S11 spotter from Scienion AG, Berlin.

4. The most important requirement on the mass spectrometer side is the possibility to run automated measurements. This should include programming the geometry of the array, alignment of the stage and the laser with the spots as well as a feature for automatic measurements. We found that all mass spectrometers tested so far including AB Sciex 4800, AB Sciex 5800 and a Bruker SolariX 9.4 T provide enough sensitivity to measure single-cell spectra. FT-MS measurements showed a decrease in signal caused by MALDI-matrix sublimation when
runtimes exceeded 6 hours.

5. Laser ablation can result in oxidation of the stainless steel surface. Incubating the chip in 15% (aqueous) acetic acid at 60°C in a sonication bath dissolves the oxidized part of the surface of the slide.

6. Proper alignment of the droplet dispensing system with the pre-structured array is essential since the MALDI-MS stage is aligned with the array geometry for MALDI-MS. Furthermore, the rough spots on the stainless steel can aid to some extent in the refocusing of drops for the matrix. Thus, we always check the alignment of a new series of slides spotting a 5 μM solution of a dye labeled fluorescent oligonucleotides into spots on the edges of the array (see Figure 6).

7. Centrifugation speed. We observed that altering the cell culture conditions by using different media resulted in variations in lysis efficiencies. Optimizing centrifugation conditions during washing the cells can mostly compensate these effects.

8. Fast freezing with liquid nitrogen is essential for two reasons: first, the fast freezing leads to quenching of the cellular metabolism. It’s important to minimize the time between the substitution of the culture medium and the freezing. Second, the freezing step greatly enhances the lysis efficiency while preventing cross-contamination on the chip.

9. Long run times (more than 6 hours) combined with MALDI matrices that undergo sublimation processes in under the vacuum conditions found in the ion source, can result in a
3.3 Methods

decrease in sensitivity during a measurement. Thus it is important to monitor the total ion counts of the analyzed spots, which should be at a constant average throughout the measurement.

10. The highly acidic conditions during crystallization of DHB convert the chlorophyll into pheophytins. Under the conditions applied this process was found to be very fast since no intact chlorophyll can be detected by MALDI-MS or by HPLC-UV.

11. The MALDI-MS measurements should generate spectra representative of the respective cells. The laser ablation of the spotted cell material should be complete, such that in a subsequent MALDI experiment on the same spot, no signals are detected. This can be validated by a fluorescence scan of the slide after the MALDI experiment that is expected to show no remaining fluorescence. If this is not the case, the cell lysis procedure should be further optimized (see Figure 7).

12. Each cell can only be measured once due to the limited amount of analytes present in a cell. For quantitative analyses of the spectra, instrumental noise should be taken into consideration. We therefore recommend only to consider peaks with $S/N > 50$. Under these conditions we found the instrumental noise to contribute less than 25% percent as tested by evaluating the isotope ratio variations (data not shown).
4 Population Screening of *Chlamydomonas reinhardtii* with Single-Cell Resolution Using a High-throughput Micro Scale Sample Preparation for MALDI Mass-Spectrometry

This chapter presents the developed method for single cell analysis in the *C. reinhardtii* wild-type strain CC-125 and a chlorophyll b knock-out strain. This chapter underlines the full strength of the method under optimized conditions (> 95 % successfully lysed and analyzed cells) and the relative quantitative nature of single-cell MALDI-MS.

*This Chapter is adapted from:*
Abstract

Consequences of cellular heterogeneity, for instance biocide persistence, can only be tackled by studying each individual in a cell population. Fluorescent tags provide tools for the high-throughput analysis of genomes, RNA-transcripts or proteins on the single-cell level. However, the analysis of lower molecular weight compounds that elude tagging is still a great challenge. Here, we describe a novel high-throughput micro-scale sample preparation technique for single cells, which provides a mass spectrum of each individual cell within a microbial population. The presented approach includes spotting of Chlamydomonas reinhardtii cells using a non-contact microarrayer onto a specialized slide and controlled lysis of cells separated on the slide. Throughout the sample preparation, analytes were traced and individual steps optimized using auto fluorescence detection of chlorophyll. The isolated sample lysate is subjected to a direct, label-free analysis using matrix-assisted laser desorption/ionization mass spectrometry. Thus we were able to differentiate individual cells of two C. reinhardtii strains based on single cell mass spectra. Furthermore we showed that only population profiles with real single cell resolution render a non-distorted picture of the phenotypes contained in a population.
Chapter 4

4.1 Introduction

Heterogeneity plays a pivotal role in the emergence of tolerance, persistence and resistance in microbial populations towards biocides [147, 148]. Also, microbial populations show highly complex interactions, e.g., in the competition for nutrients or in the colonization of new habitats [149]. In recent years, newly developed tools for single-cell analysis have greatly extended our understanding of biological variation in microbial populations and its underlying mechanisms [3]. These tools allow genome sequencing [150] or follow transcription as well as protein synthesis on the single-cell level [151]. Since these techniques give a much higher resolution when observing processes in given cell populations, they permit insight into inter- and intra-cellular processes and the underlying mechanisms. However when it comes to high-throughput measurements of small molecules, very few methods are known as of now [137].

Singularities of individual cells can only be fully appreciated within the context of the population. Therefore, one of the most important features for single-cell methods to be useful in biological applications is high-throughput capability. One of the most successful high-throughput approaches to characterize heterogeneities in microbial populations is flow cytometry [152]. It benefits from high sensitivity and a high linear dynamic range of fluorescence tagging and optical detection. However, the method is strongly limited in parallelization, since excitation and emission bands overlap. Mass cytometry on the other hand, which is a new approach that can be coupled to flow cytometry, uses antibodies tagged with rare earth metals [153]. With mass spectrometric detection, over 40 features can be measured in each cell, implying that mass spectrometry (MS) can boost parallelization capabilities [81].

Unfortunately, labeling small molecular compounds is not possible, because the chemical behavior of low molecular weight compounds is heavily affected by the tagging. Furthermore, the selectivity of tags is often determined by the strength of non-covalent
interactions specific to an analyte, which can render labeling strategies highly complex. Low molecular weight compounds often lack enough distinctive binding motives to allow for specific binding in complex cellular environments.

On the other hand, some analytes (e.g. molecules with structural functions such as lipids) are present in much higher copy numbers inside cells compared to DNA or proteins, such that they are within the reach of state-of-the-art mass spectrometric detection as was shown in recent years [154, 155]. Matrix-assisted laser desorption-ionization (MALDI) imaging mass spectrometry is the method of choice for the analysis of tissues [156] and new laser desorption sources render it possible to image the distribution of small molecules with single-cell resolution [136, 157]. Together with its high-throughput capabilities single-cell MALDI-MS can give new insights into cell-to-cell variations of low molecular weight compounds. However the applicability of these methods to microbial colonies is a big challenge due to the necessity of harsher extraction conditions and the significantly smaller cell size [132].

Here we present a new method for single-cell sample workup of microbial cell cultures and their discrete analysis using MALDI-MS. Our method targets the analysis of individual cells in suspension e.g. cells of microbial cell cultures. As exemplified for the alga *C. reinhardtii*, the workflow presented comprises a controlled sample preparation protocol for thousands of individual cells in spatially separated microarray spots, that allows the reproducible extraction and analysis of microbial cells - despite their robust cell walls [98]. Endogenous chlorophyll was detected by MS and by fluorescence measurements. Thereby, each sample preparation step could be monitored optically for optimization. Finally, MS allowed a clear differentiation of the two strains of *C. reinhardtii*, based on data obtained for each single algae cell. The necessity of true high-throughput single-cell measurements is demonstrated by the deterioration of characteristic signatures of the two strains when considering multi-cell spots.
4.2 Materials and Methods

4.2.1 Chemicals

All solvents were purchased in HPCL grade quality. Acetone (Chromasolv), chloroform (ReagentPlus) and isopropanol (Chromasolv) were purchased from Sigma-Aldrich. Water (LiChrosolv) was purchased from Merck. The MALDI-matrix 2, 5-dihydroxybenzoic acid (DHB) was purchased from Sigma-Aldrich. Hutner's trace elements were purchased from the Chlamydomonas resource center (St.Paul, MN, USA).

4.2.2 Cell Culture

*Chlamydomonas reinhardtii* strains CC-125 (wild-type) and CC-4346 (chlorophyllide a oxidase mutant [158]) were obtained from the Chlamydomonas Resource Center, St. Paul MN, USA. Strains were maintained on tris-acetate-phosphate (TAP) agar plates. For single-cell MS single colonies of strains were inoculated in liquid TAP medium [159]. The cells were cultured in an incubation shaker (Minitron, Infors HT) at 21°C at 110 rpm and 1200 lux continuous illumination.

4.2.3 Characterization of *C. reinhardtii* strains

The *C. reinhardtii* strains were characterized on population level using UV-VIS spectrometry. Absorption was measure between 350 nm and 850 nm. In UV-VIS spectra, the CAO insertional mutant strain CC-4346 lacks the characteristic shoulder peaks caused by chlorophyll b absorption around 660 nm and 500 nm.

4.2.4 Microarrays

The Microarray for Mass Spectrometry (MAMS) targets were prepared as follows. Stainless steel target slides 25 mm x 75 mm were structured using picosecond laser pulses. Before usage, a slide was cleaned using a sequence of different solvent conditions: 1. chloroform 2. acetone 3. isopropanol 4. 15% acetic acid in water 5. water. Each of
the conditions was maintained for at least 60 min in a sonication bath. The slides were stored under a nitrogen atmosphere until use.

4.2.5 Spotting the cells onto microarray slides

For liquid handling of nanoliter volumes a spotting robot (sciFLEXARRAYER S11, Scienion) was used. For single-cell MS, _C. reinhardtii_ strains were centrifuged just before spotting at 2000 x g / 1500 x g for 5 min (Eppendorf 5415R centrifuge) and 3 times resuspended in deionized water to reduce the quenching influence of salts contained in the TAP liquid culture medium. After spotting the cells, the slide was scanned using a confocal microarray scanner (LS400, Tecan). Fluorescence was excited at 633 nm and detected at 670 nm. The detector gain was at kept constant for all measured slides for comparing relative intensities. Scan resolution was 6 μm.

4.2.6 Cell lysis and MALDI-matrix application

To enhance extraction and co-crystallization of the analytes with the MALDI-matrix (2,5-dihydroxybenzoic acid) while avoiding cross-contamination between microarray spots the slide was submerged in liquid nitrogen. The slide was reconstituted to room temperature in a desiccator to avoid condensation. A fluorescence scan of the slide was performed after reconstitution to check for possible cell displacements or cross contamination. After this step 2,5-dihydroxybenzoic acid (10 mg/ml in 80% aqueous acetone) was spotted onto the cells to extract the analytes and co-crystallize them with the MALDI matrix. The efficiency of the extraction was monitored with a confocal fluorescence scan. The slide was stored under nitrogen atmosphere at -80° C until measured.

4.2.7 MALDI-FT-ICR-MS

Accurate mass spectra of single cells and cell bulk was recorded using a FT-ICR mass spectrometer using the MALDI source Bruker SolariX 9.4T. Additionally MS/MS spectra were recorded using
collision-induced dissociation. The laser was operated using a large laser focus (≈100um) and a laser power of 78% arbitrary units (a.u.), with 200 laser shots over 10 scans.

### 4.2.8 MALDI-TOF-MS

High-throughput single-cell MS was performed using a commercial MALDI mass spectrometer (ABSciex 5800). The laser intensity was set to 4300 a.u. with a laser repetition rate of 200 Hz and extraction time delay of 500 ns. A total of 70 sub-spectra were recorded for each spot ablating in a spiral pattern. The MALDI method used had a mass resolution of 9200 (±750) for chlorophyll a, and a mass accuracy of ±10ppm.

### 4.2.9 Differentiating strains

To generate distinct profiles for the two *Chlamydomonas reinhardtii* strains CC-4346 [158] and CC-125 the two strains were spotted from separate cultures, each strain covering half of a microarray slide. Spotting a mixture of the strains covered a second microarray. Using a higher cell concentration in the mixture, the second microarray contained a higher number of spots with multiple cells.

### 4.2.10 Statistical analysis of mixed strains

The number of cells for each spot was determined using fluorescence images of the microarray (ex. 670 nm / em. 630 nm). The spectra were divided into groups depending on the number of cells in the spot. The distribution of the chlorophyll b/ chlorophyll a ratio was fitted using a kernel density estimation using the dfittool from MATLAB version 2014a (normal, automated bandwidth). The probability density function was then fitted with Gaussian distributions accounting for the contribution of the wild-type strain CC-125. Since the chlorophyll b/a ratio in strain CC-4346 is zero due to the complete absence of chlorophyll b, the averaged signal of the two strains is fitted with a Gaussian distribution.
4.2 Materials and Methods

Figure 8 High-throughput microscale sample preparation for single-cell MALDI-MS. (A to D) Schematics of the individual steps of the workflow. (E to H) Confocal fluorescence scans of 9 wells on the microarray. (A, E) The cleaned, empty microarray slide has wells where the stainless steel surface is roughened up. (B, F) Cells are spotted from cell suspensions. Chlorophyll autofluorescence makes the cells clearly visible. (C, G) Fast freezing of the cells in liquid nitrogen leads to cell wall damage. (D, H) Chlorophyll and other soluble analytes are extracted from the cells by the matrix solution (10 mg/ml DHB in aqueous acetone). Evaporation of the solvent leads to cocrystallization of the extracted analytes with the MALDI matrix.
4.3 Results

4.3.1 Single-cell sample preparation of microbial cells.

Extracting the small amount of molecules from a microbial cell, which is surrounded by tough cell walls, is challenging. Most importantly the extraction has to reproducibly extract a representative fraction of the contents of each cell. A schematic and fluorescence scans of the key steps during sample preparations are presented in Figure 8. To minimize cross-contamination between adjacent spots on the microarray the liquid handling was carried out using a piezo-driven contactless spotting device as described in the experimental section. The microarray slide has 1430 spots of 300 μm diameter each and 720 μm center-to-center distance. A detailed protocol of the spotting procedure can be found in TableS1. A very low number (2/1430) of false positive spectra, i.e. spots featuring a mass spectrometric signal reminiscent of a cell without a cell being present, indicated that cross-contamination is indeed negligible. Thanks to the orthogonal fluorescence detection of the auto-fluorescent chlorophyll it can be visualized throughout the sample preparation process. Fluorescence detection allowed the monitoring of the lysis and extraction of the cells. Moreover, contamination caused by early lysis in the medium or during the analyte extraction can be optically traced back. Our improved lysis procedure not only increased the percentage of successful single-cell measurements in MALDI-TOF-MS to about 95% for the wild-type CC-125, but also boosted the signal-to-noise value of chlorophyll a in the spectra to a median value of 540.

4.3.2 High-resolution MALDI-MS for peak identification.

To assign the signals obtained in single-cell measurements using MALDI- (TOF-) MS on Chlamydomonas reinhardtii, we used MALDI-FT-MS to determine their accurate mass (≤ 0.5 ppm mass deviation) and performed population level MS/MS measurements (see Appendix, Chapter 8.1, Figure S 1) which were matched with Chlamydomonas
reinhardtii literature [100, 160] allowing for high confidence signal identification. A table of assigned compounds can be found in the Appendix 8.1 Table S 2. Using the MALDI matrix DHB in the positive ion mode, the peaks identified from single-cell spectra can be assigned to different lipid classes such as monogalactosyl-diacylglycerols (MGDG), digalactosyl-diacylglycerols (DGDG), homoserine lipids (DGTS) or to chlorophylls. Chlorophylls are detected in the form of pheophytins due to the acidification of the extract during crystallization with the acidic DHB. The acidification leads to complete dissociation of Mg\(^{2+}\) from the porphyrin ring and therefore to the formation of the corresponding pheophytins [161]. No intact chlorophylls are detected. Other major thylakoid lipid constituents such as sulfo- as well as phospholipids can be detected on the single-cell level in negative mode spectra using 9-aminoacridine (data not shown). Single-cell mass spectra show the same peak composition as spectra collected from a bulk of cells but they differ in relative composition (see Figure 9).
Figure 9 MALDI mass spectra obtained from a single *Chlamydomonas reinhardtii* cell and from cells in bulk on an FT-MS instrument. The single-cell mass spectrum is background corrected for better visibility of the peaks in the lower mass region. This was achieved by subtracting the spectrum of an empty spot from the single-cell spectrum. Rel. Int., relative intensity; Car, carotenoids; Chl, chlorophyll.
4.3 Results

4.3.3 Characterization and identification of strains on the single-cell level.

We used the new technique to distinguish two different strains of *Chlamydomonas reinhardtii* at the single-cell level. The distinction between the two strains was based on the presence and absence of a single compound, chlorophyll b: the wild-type strain CC-125 contains both chlorophyll a and b, while the chlorophyllide-a-oxidase insertional mutant CC-4346 contains chlorophyll a but lacks chlorophyll b [158]. The absence of chlorophyll b in the mutant strains was verified both by MALDI-MS and by UV-VIS measurements on the population level (see Appendix 8.1, Figure S 2). Single-cell spectra were recorded producing a population profile for both strains (see Appendix 8.1, Figure S 3). The cells were spotted and lysed on the array slide as described above. 715 spots of each cell suspension were spotted. In 541 spots a single cell from strain CC-125 was found by fluorescence detection, 511 of these spots showed chlorophyll a signals in MALDI-MS. Single cells of strain CC-4346 were present in 332 spots with 283 of these showing a signals of chlorophyll a in MALDI-MS. The raw spectra are plotted as a heat map (MATLAB, msheatmap function, midpointValue = 0.99) in

**Figure 10.** The background peaks, caused by the MALDI matrix or contaminants are stable throughout the 1430 spectra, as clearly visible from the heat map. Furthermore, the strains can be easily distinguished based on the absence of chlorophyll b in CC-4346.
Figure 10 Single-cell mass spectra obtained from one measurement series. Spectra originate from two different strains of *Chlamydomonas reinhardtii*. CC-125 is a wild-type strain, while CC-4346 is a chlorophyll b-deficient mutant strain, reflected by the absence of the pheophytin b signal at 885.6 m/z. The visualization was achieved by using the `msheatmap` function in MATLAB. The midpoint value chosen was 0.99, which means that only the most intense 1% of signal values appear in the representation.
4.3.4 Deterioration of strain-specific phenotypes in multi-cell mass spectra.

A key advantage of any single-cell resolved technique is the distinct information it can supply in contrast to population measurements. Therefore we performed an experiment with an up concentrated cell suspension to see how the population profile differs when looking at single cells compared to multi-cell spots. Interestingly, single-cell measurements from this run could be assigned to one of the two strains solely based on the mass spectrum. This is the first report on strain identification in single cells based on MS. Furthermore, spots that contain more than one cell either show distinct or mixed responses that can be attributed to contributions wither one or both strains. The more cells per spot the higher is the chance for a superimposed readout as shown in Figure 11. This emphasizes the ability of the method to perform real single-cell measurements and the detrimental effect of even few cell measurements on the observed population heterogeneity.
Figure 11  Probability densities of the chlorophyll b / chlorophyll a peak area ratios for spots containing 1 to 5 cells (dark blue trace). Single-cell distributions are averaged out (yellow trace) as the number of cells per measured spot increases. When single cells of the chlorophyll b-deficient strain CC-4346 (red) are measured, the chlorophyll b/chlorophyll a peak ratio is zero, while wild-type strain CC-125 (green) shows a ratio of around 0.1. The two strains can therefore easily be distinguished on the single-cell level. Measuring spots with more than one cell from mixed cultures leads to an intermediate response due to averaging of the two strain-specific responses. The effect is more pronounced the more cells are measured in one spot.
4.4 Discussion

MALDI-MS of single cells allows the detection of abundant low molecular weight compounds with polar character. The analytes identified from single-cell measurements using a widely used matrix (DHB) were mainly lipids belonging to different classes such as thylakoid membrane lipids, membrane lipids, or pigments. The selectivity for these compounds can be attributed to their abundance, polarity and solubility in the extractive solvent. Application of the MALDI matrix, which is dissolved in a solvent mixture, represents a liquid extraction from the cracked cells and helps to reduce the complexity of the biological background. Changing the extracting solvent or the matrix therefore allows access to different analytes and changes their relative contribution to the obtained cell spectra. For maintaining single-cell resolution preventing cross-contamination between spots is essential. Both the non-contact microarrayer and the spacing of the microarray help to avoid cross-contamination. Only 2 of 453 empty wells showed MS signals reminiscent of cells in their spectra.

Another aspect of utmost importance for successful single-cell analysis is cell lysis. If the lysis process is not effectively controlled, the developmental state or nature of the cell wall will strongly influence the extraction. Thus, a bias for cells with a weakened cell wall due to changes in cell wall composition, during, e.g., the cell cycle or aging would be introduced. Incomplete lysis can result in poor or irreproducible mass spectrometric readout, since the MALDI favors the ionization of the fraction “co-crystallized” with the matrix. Our system gives experimental evidence of this phenomenon, since fluorescence scanning after MALDI-MS shows significant fluorescence intensity for non-lysed cells (see Appendix 8.1, Figure S 4). The orthogonal fluorescence information thus not only helped in optimizing the method but also helps to retrace the sources of errors. The optimized lysis procedure also improves spectral quality by increasing the percentage of single cells that yield a spectrum, the signal-to-noise values and the number of detected analytes.
In many applications, MALDI-MS performs best giving qualitative information. However, relative quantitative information can be obtained when biological matrix background is reproducible. Since our method is label-free, it delivers direct access to the phenotype of individual cells. The composition of small molecules in individual cells of microbial populations can be used, e.g., to determine the penetrance of a mutation on the single-cell level. We therefore used the methods to differentiate cells of different strains based on single-cell mass spectra. *Chlamydomonas reinhardtii* mutant CC-4346 that is incapable of chlorophyll b biosynthesis [158] can be differentiated from the wild-type by the absence of chlorophyll b. As predicted by population level MALDI-MS as well as UV-VIS spectroscopy, there were no detectable levels of chlorophyll b in the knockout even when analyzed with single-cell resolution. Mixing the wild-type and the chlorophyll-b-less mutant strain still showed distinct phenotypes in a mixed population. This was underlined by analyzing multi-cell measurements of the mixture of the two strains. Mixing the two strains caused a rapid deterioration of the distinct spectral response from the two strains with increasing numbers of cells measured, since both strains contribute to the spectra. This is an important validation of the experimental data that highlights the importance of real single-cell spectra for the characterization of molecular phenotypes present within a population. Since our experimental system represents the an extreme case where the distinction is based on the presence or absence of a signal, this effect is expected to be more severe if the differences of the two phenotypes in the population are less pronounced i.e. when distinctions are based on different amounts of a compound.

While recent publications still included few-cell spectra [155], the newly developed high-throughput sample-preparation method, allows to measure pure single-cell profiles from microbial populations and their mixtures for the very first time. The quality and the flexibility of the single-cell measurements are highly promising concerning future applications of MALDI-MS for single-cell resolved population analysis in microbiology.
5 Single-cell MALDI tandem-mass spectrometry: unambiguous assignment of small biomolecules from single *Chlamydomonas reinhardtii* cells

This chapter reports a significant improvement in the assignment of signal detected in single cells. The assignment of peaks in single cells mostly relies on bulk extract measurements. The use of tandem mass spectrometry directly in single cells, allowed us to generate characteristic fragment ions directly from single cells which renders a significant boost to the certainty of compound assignment in single-cell mass spectra.

*This Chapter is adapted from:*
Abstract

The analysis of compounds from single cells is a major challenge in analytical life science. Labeling strategies such as fluorescence detection are well established for measuring proteins with single cell sensitivity, but they mostly fail to detect small molecules. More recently mass spectrometry has entered the realm of single cell sensitivity and enables the label free and highly parallelized detection of small biomolecules from single cells. The assignment of signals detected in single cells, however, generally has to rely on measurements in whole cell culture extracts. Isobaric structures, contaminations, higher noise levels and the high variability in the abundance of peaks between single cells complicate the assignment of peaks in single-cell spectra. Tandem mass spectrometry would be very useful for compound identification via mass spectrometry directly in single-cell analyses. Here we present the first single cell tandem mass spectra collected using matrix-assisted laser-desorption/ ionization. The spectra obtained allow the assignment of most compounds detected in the spectra. We also show that the fragmentation is not restricted to the most abundant peaks in the spectra, but over a dynamic range of more than one order of magnitude.
5.1 Introduction

Individual cells are the smallest functioning unit of life and a key challenge in analytical life science. The analytical scientist is challenged by high sensitivity and selectivity required by for the analysis single cells [162]. The biologist, on the other hand, seeks to understand the individual cell within the biological context of its population or tissue, which adds high throughput to the list of challenges for analytical methods to overcome. There are plenty of single-cell techniques in the area of DNA [58, 163], RNA [164, 165] and protein analysis[72, 166] that have found their way into biologist’s labs. These techniques benefit from the amplification tools adopted from molecular biology and the high linear dynamic range and sensitivity of fluorescent probes.

However few such tools exist for the analysis small molecules from single cells [167], despite the fact that there is a great need for phenotypic and metabolic characterization of single cells, especially since changes in genomes and transcriptomes are often poor predictors of cellular phenotypes [168]. Mass spectrometry has made great progress with respect to sensitivity and resolution in the past decades.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [169] in particular can provide both the high throughput and the sensitivity needed for the analysis of large numbers of single cells [170]. Analyzing complex and dynamic biological systems such as cells, however, requires not just detection of masses but also the assignment of the signals detected to functionally relate these to biological processes.

Tandem mass spectrometry is an important part of the methodological toolbox of a mass spectrometrist for the several reasons [171]. First, different constitutional isomers can be attributed to a single isobaric signal, which makes it necessary identify molecules using characteristic fragments. Second, contamination from different sources like solvents, the cell culture medium, cell manipulating devices or the MALDI-matrix can give rise to interferences that show the same or highly similar masses. This effect is especially important in single-
cell mass spectrometry because the weight of single cells is in the range of $10^{-12}$-$10^{-15}$ grams [172, 173]. Third there is great variability in the relative abundance of molecules between single cells, which can complicate the peak assignment. Unfortunately the MS/MS capabilities of mass spectrometry approaches for single cell analysis have very much lagged behind.

Recently we reported a method for the high-throughput analysis of single cells for *Chlamydomonas reinhardtii*, a green freshwater algae and a well-studied model organism in photosynthesis and biofuel research [142]. The method allowed the parallel detection and relative quantitation of more than 20 assigned peaks from different lipid classes with high throughput. MS/MS capabilities would greatly enhance the explanatory power based on higher confidence in peak assignment.

Here, we present the first ever MS/MS spectra collected from single cells using MALDI as an ionization method. The cells were analyzed using 2,5-dihydroxybenzoic acid (DHB) as a matrix, in positive ion mode. The method allowed us to assign about 15 compounds in our spectra belonging to different lipid and pigment classes. The assignments based on the single cell tandem mass spectra are in good agreement with spectra obtained by analyzing lipid extracts, as well as the relevant molecular biology literature [100, 174, 175].
5.2 Experimental Section

5.2.1 Chemicals

2,5-dihydroxybenzoic acid (DHB) was purchased from Sigma-Aldrich. Chlorophyll a (from *Anacystis nidulans*), chloroform (> 99.8%), acetone (puriss. p.a., ≥99.5%) and 2-propanol (puriss., ≥99.5%) were purchased from Sigma Aldrich, Switzerland. Water (Optima® LC/MS grade) was purchased from Fisher-Chemicals. Hutners trace element solution for cell culture media preparation was obtained from the Chlamydomonas Resource Center (St. Paul, MN, USA).

5.2.2 MS/MS of lipid extracts

A lipid extract was performed with the Bligh-Dyer method [176] using *C. reinhardtii* wild-type strain CC-125 obtained from the Chlamydomonas Resource Center, St Paul, MN. The cells were cultured in tris-acetate-phosphate medium [159]. To aid the extraction the cells were sonicated for 30 seconds. The chloroform phase was collected and stored at -20°C. Then the chloroform was evaporated and the lipids solubilized in aqueous acetone (80 vol% acetone, 20 vol% water). The samples were mixed with DHB (10mg/ml in 80% aqueous acetone) on the MALDI target (384 Opti-TOF 123mm x 61 mm SS, ABSciex, Toronto) in a sandwich fashion (0.5 μl DHB – 0.5 μl lipid extract – 0.5 μl DHB). The MS/MS method used for extracts was identical to the one described below.

5.2.3 Sample Preparation for single-cell MS/MS

Wild-type *C. reinhardtii* cells (strain CC-125) were centrifuged (3500 x g for 5 min) three times and resuspended in water. The cells were placed on a micro-structured array for MALDI mass spectrometry as reported previously [142]. In short, the array consists of 55 x 26 spots of 300 μm diameter each spaced by 720 μm on a stainless steel plate. A layer of matrix was added first, followed by deposition of the washed cells into the spots (Figure 12 A). Cellular metabolism was
quenched immediately by immersing the entire array into liquid nitrogen. Extraction of analytes and co-crystallization with the matrix is achieved by recurrent extraction adding 5 x 5 nl of 10 mg/ml of DHB dissolved in 80% aqueous acetone and letting the spots crystallize after each step. Fluorescence scans 630 nm excitation and 670 nm emission were collected of the blank slide, of the cells after quenching, and after matrix application.

**Figure 12** Sample preparation procedure for single-cell MS/MS. Chlorophyll autofluorescence is measured at 630 nm excitation and 670 nm emission. Both of the images are scaled identically for better visibility (0-3000 instead of 0-65000) and falsely coloured. The diameter of a spot is 300 μm. Cells are around 10 μm but appear larger due to over-scaling and scattering. **A** The autofluorescent cells are imaged on the slide prior to lysis to determine the number of cells in each spot. As shown in the graphical abstract below a layer of matrix is applied prior to applying the cells. **B** Scanning the slides after co-crystallization can monitor the success of cell lysis. Matrix autofluorescence is negligible. Only spots containing well-lysed cells should be used for single-cell MS/MS experiments.
5.2 Experimental Section

5.2.4 Single-cell MS/MS

The tandem mass spectra were recorded on a reflectron MALDI-TOF instrument (AB Sciex 5800, Toronto, CA). The spots for MS/MS were selected from the plates based on the fluorescence scans. Spectra were collected from spots showing single cells in the fluorescence scan after quenching. To confirm the fact that the signal is produced only in the presence of cells MS/MS were recorded for the same precursor mass on spots showing no cells. In none of the cases we detected fragments of cellular analytes in the absence of cells. The MALDI parameters used were: a delayed extraction time of 300 ns, a laser intensity of 5500 a.u. and a laser repetition rate of 1000 Hz. In all cases spectra were collected with and without metastable suppression. Precursors were isolated using a mass window of ±1.5 Da, using the QuanTis timed ion selector of the reflectron TOF system operated with a TIS offset of 1.5 mm.

5.2.5 Data analysis

Peak picking was performed according to a S/N ≥ 10 criterion. Spectra were calibrated externally using chlorophyll a as a reference. The spectra were smoothed using the default settings in the Data Explorer software (ABSciex, Toronto).
5.3 Results and Discussion

The tandem mass spectrometry method was optimized on a chlorophyll a standard and a lipid extract of *C. reinhardtii*. The lipid composition of *C. reinhardtii* is well studied, since the species is both a model organism in photosynthesis and a candidate for biofuel production. From an experimental perspective one of the key advantages in using the algae as a model for optimizing and developing a single cell method is that due to its autofluorescent pigments in the form of chlorophylls it is possible to visualize cell lysis, a key step for successful single cell mass spectrometry [142]. Two aspects are very important here: the first is to make sure that the actual measurement is taken on a single cell. In the current procedure the cells are placed on a microarray by spotting a cell culture at an optimized cell density. This procedure leaves most spots occupied with single cells, which can be identified, based on fluorescence scans (Figure 12 A). However, some spots remain empty while multiple cells occupy others. These spots are discarded for analysis. The second key step is lysis and co-crystallization (Figure 12 B). Only spots that show well-lysed cells as shown in Figure 12 are considered for MS/MS. Poorly lysed cells may still show signals in MS mode, but these are generally too low in intensity to perform MS/MS experiments. Relying on the sample preparation protocol for single-cell MALDI optimized for *C. reinhardtii* we were able to assign 16 compounds to 14 mass spectral peaks (Table 1) by performing single-cell MS/MS measurements. The assignments are backed up by literature. The most prominent signal in the single cell spectra is the pigment chlorophyll a. However due to the presence of DHB, which is an acid, the extraction of the lipids is carried out under low pH conditions. This leads to a release of the Mg$^{2+}$ ion from the porphyrin macrocycle. Chlorophyll a is therefore detected as pheophytin a ([M-Mg$^2$+3H]$^+$ = 871.57 Da) using DHB as a matrix [175, 177]. This is also true for chlorophyll b ([M-Mg$^2$+3H]$^+$ = 885.55 Da). The single-cell MS/MS of chlorophyll a is dominated by the m/z = 593.28 Da due to the loss of the phytol chain (Figure 13A and 13D). Chlorophyll b
fragmentation leads to the formation of 607.25 Da fragment due to phytol loss. Since chlorophylls are absorbing the wavelength of the MALDI laser at 355 nm both CID as well as in-source decay contribute to fragmentation. An increase in laser intensity was found to enhance fragmentation even in the absence of CID gas, which supports this interpretation (data not shown).

Furthermore, the spectra show all of the most abundant lipids, which can be detected in positive ion mode, both form the plastidic, i.e. of organellar origin and extraplastidic membrane systems. Plastidic

![Figure 13](image)

**Figure 13** Single-cell MS/MS and corresponding chemical structures of different compound classes detected in single cells. **A/D** Single-cell MS/MS of chlorophyll a the structure showing the fragmentation explaining the most abundant signals in the MS/MS spectra. **B/E** SC MS/MS and the chemical structure of MGDG a plastidic galactolipid. **C/F** SC-MS/MS and chemical structure of a DGTS, an extraplastidic lipid. The highly stabilized charge leads to the detection of the lipid headgroup even in single-cell spectra. The positions of the two fatty acids (sn1 or sn2) cannot be determined using MS/MS but are adapted from literature.
membrane systems that include the thylakoid membranes, in which the photosynthetic complexes are embedded, mainly consist of the galactolipids mono- and digalactosyl-diacylglyceol (MGDG and DGDG) and sulfolipids in the form of sulfoquinovosyl-diacylglycerol (SQDG) [100]. Since the extraction protocol was optimized for chlorophyll, it is reasonable that the major membrane constituents are co-extracted. MGDG and DGDG can be detected in the form of sodium ([M+Na+]⁺) or potassium ([M+K+]⁺) adducts using MALDI-MS as previously reported by Vieler et al [177]. In the single cell mass spectra the sodium adducts are most prominent.

The SC-MS/MS spectra of both MGDG (see Figure 13B and 13E) and DGDG lipids are characterized by neutral loss of the fatty acid side chains [178]. Despite the fact that MGDG (16:4/18:3) makes up for almost 80% of the MGDG lipid, which in turn contribute about 50% of the total thylakoid lipid, the relative intensity of the peak is relatively low at 18%. This can be attributed to a lower ionization efficiency due to the sodium adduct formation.

The major extraplastidic lipid classes are the diacylglycerlytrimethylhomoserines (DGTS) which functionally replace phosphatidylcholines in C. reinhardtii [100]. The DGTS lipids show a great variety of fatty acid side chains in the single-cell spectra (Table 1, Figure 13C and 13F). One reason for this lipid class to be readily observed might be the high ionization efficiency of the highly polar lipid headgroup. The trimethylhomoserine-headgroup itself as well as various fragments generated by the loss of fatty acid side chains can also be detected in single cell MS/MS spectra (see Figure 14). In contrast to the galactolipids the side chain fragmentation leads both to the fatty acid neutral loss and the cleavage of the acyl group with the latter being more prominent in the single cell MS/MS spectra. The relative abundance of the different DGTS species agrees well with previously reported values [174]. A unique advantage of applying MS/MS is that fragmentation of the side chains allows the assignment of multiple isobaric species. We were able to assign multiple isobars for the signal at 756.57 Da (DGTS (18:2/18:2) and DGTS (18:1/18:3)) and 760.61 Da
(DGTS (18:3/18:3) and DGTS (18:4/18:2)) respectively. One explanation for the detection of multiple isobars in the case of 756.57 Da and 760.61 Da is the similar abundance of the two contributing DGTS species [174].

**Figure 14** Precursor ion suppressed single-cell MS/MS from two DGTS lipids. The DGTS at 732.58 Da (upper spectrum) fragments to 474.32 Da and 494.33 Da corresponding to the acyl loss of the fatty acids 18:4 and 16:0 respectively. DGTS at 734.59 Da (lower spectrum) fragments to 474.32 Da and 496.36 Da corresponding to the acyl loss of 18:3 and 16:0 respectively.
Table 2 List of compounds assigned by SC-MS/MS in *Chlamydomonas reinhardtii* using DHB in positive ion mode. Relative intensities relate to single-cell MS spectra collected from the same population. Numbers in brackets relate to the fatty acid composition (carbon number: number of double bonds) the position of the fatty acid or the position of the double bonds cannot be determined using single-cell MS/MS.

<table>
<thead>
<tr>
<th>metabolite name</th>
<th>rel. int.</th>
<th>species detected</th>
<th>parent mass</th>
<th>main fragment</th>
<th>other fragments</th>
</tr>
</thead>
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<td>Pigments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>100</td>
<td>[M-Mg^{2+}+3H]^+</td>
<td>871.57</td>
<td>593.28</td>
<td>533.25, 519.30</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>34</td>
<td>[M-Mg^{2+}+3H]^+</td>
<td>885.55</td>
<td>607.25</td>
<td>547.24,</td>
</tr>
<tr>
<td>Galacto-lipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGDG (16:4/18:3)</td>
<td>18</td>
<td>[M+Na]^+</td>
<td>767.47</td>
<td>489.24</td>
<td>519.28</td>
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<tr>
<td>DGDG (18:3/16:3)</td>
<td>19</td>
<td>[M+Na]^+</td>
<td>931.53</td>
<td>653.27</td>
<td>681.28</td>
</tr>
<tr>
<td>DGDG (18:2/16:3)</td>
<td>15</td>
<td>[M+Na]^+</td>
<td>933.55</td>
<td>653.28</td>
<td>683.45</td>
</tr>
<tr>
<td>DGDG (18:3/16:0)</td>
<td>9</td>
<td>[M+H]^+</td>
<td>937.58</td>
<td>659.31</td>
<td>681.22</td>
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<tr>
<td>DGDG (18:2/16:0)</td>
<td>9</td>
<td>[M+H]^+</td>
<td>939.6</td>
<td>659.3</td>
<td>683.24</td>
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<tr>
<td>Homoserine lipids</td>
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<tr>
<td>DGTS (16:0/18:4)</td>
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<td>[M+H]^+</td>
<td>732.58</td>
<td>474.33</td>
<td>494.30, 236.16</td>
</tr>
<tr>
<td>DGTS (16:0/18:3)</td>
<td>49</td>
<td>[M+H]^+</td>
<td>734.59</td>
<td>474.37</td>
<td>496.38, 236.16, 474.37, 456.34</td>
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<tr>
<td>DGTS (16:0/18:2)</td>
<td>21</td>
<td>[M+H]^+</td>
<td>736.61</td>
<td>474.36</td>
<td>498.33</td>
</tr>
<tr>
<td>DGTS (18:3/18:4)</td>
<td>19</td>
<td>[M+H]^+</td>
<td>754.55</td>
<td>496.42</td>
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<td>DGTS (18:2/18:3)</td>
<td>26</td>
<td>[M+H]^+</td>
<td>758.6</td>
<td>496.36</td>
<td>498.39</td>
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<tr>
<td>DGTS (18:2/18:2), DGTS (18:1/18:3)</td>
<td>18</td>
<td>[M+H]^+</td>
<td>760.61</td>
<td>498.36</td>
<td>496.45, 500.38</td>
</tr>
</tbody>
</table>
5.4 Conclusions

Peak assignment in single cell spectra is a challenge for the advancement of single-cell mass spectrometry techniques. The certainty of the actual assignment based on fragment detection – despite all database searches and high mass accuracy – is unsurpassed. The successful implementation of MS/MS experiments on the single-cell level as shown here reflects the progress made with respect to sensitivity and reproducibility, and adds an important amendment to single-cell MALDI mass spectrometry.
6 Single-cell Mass Spectrometry Reveals the Importance of Genetic Diversity and Plasticity for Phenotypic Variation in Nitrogen Limited *Chlamydomonas*

This chapter constitutes the application of the single cell mass spectrometry method in a batch growth time series experiment of different genetically diverse and isoclonal nitrogen limited *C. reinhardtii* cultures. By measuring phenotypic variation in lipid composition we were able to monitor the response and adaptation of the population with single-cell resolution.

*This chapter was adapted from:*
Krismer, J., Tamminen, M., Zenobi, R., Narwani, A. “Single-cell mass spectrometry reveals the importance of genetic diversity and plasticity for phenotypic variation in nitrogen limited Chlamydomonas”, *submitted to ISME Journal*
Abstract

Phenotypic variation is vital for microbial populations to survive environmental perturbations. Both genetic and non-genetic factors contribute to phenotypic variation. To investigate the correlation between genetic diversity and phenotypic variation, we applied our recently developed mass spectrometry method that allows for the simultaneous measurement of more than 25 different lipids and pigments with high throughput in the unicellular microalga *Chlamydomonas reinhardtii*. We monitored the impact of nitrogen limitation on a genetically diverse wild-type strain CC-1690 and two clonal isolates from CC-1690 named ANC3 and ANC5. Measuring molecular composition of thousands of single cells at different time points of the experiment allowed us to capture a dynamic picture of the phenotypic composition and adaptation of the populations over time. Phenotypic variation in the generically diverse culture CC-1690 was maintained over the whole time course of the experiment, which indicates differences in the cells susceptibility towards N-limitation. Notwithstanding their different phenotypic responses, individual cells of both isoclonal cultures showed higher synchronicity in their phenotypic response compared to CC-1690. ANC3 showed high chlorophyll levels under nitrogen replete conditions, which resulted in a high fraction of stressed cells on day 9, while ANC5 showed a ‘play-it-safe’ strategy with low chlorophyll levels, resulting in a lower fraction of stressed cells on day 9.
6.1 Introduction

The ability of individual organisms to adapt to their environment is a basic feature of life. Upon change in environment, phenotypic variation between individuals of a population can result in adaptive or non-adaptive phenotypes [179]. In the face of rapid or unpredictable environmental change, phenotypic variation can therefore increase the average fitness of individuals in a population, whereas, in a stable environment, phenotypic variation can decrease average fitness [180-184]. Phenotypic variation of organisms can result from genomic differences among individuals but is also found in isoclonal populations, e.g. phenotypic plasticity and heterogeneity [40, 181]. Therefore, both genetic and non-genetic differences between cells contribute to the phenotypic variation within a population. Understanding their importance is pivotal to elucidate their impact on ecology and evolution [179, 185]. The study of laboratory evolution in microorganisms has many advantages, among which the ease of manipulating environmental factors and the short replication time are key [20]. Unfortunately, the majority of our knowledge on phenotypes of unicellular organisms is generated by measuring population-level rates and averages. This is an issue especially in studying evolution, since it is not possible to predict how a population will respond to selection in new environments without understanding the extent of single-cell level phenotypic variation within a population; further, it is not easy to determine how much of this response is contributed by plasticity, and how much is genetically determined [180]. The informative value of a population average for understanding the behavior of individual cells in a population depends on the extent and structure of phenotypic variation [7].

While there is a wealth of recently developed methods for extracting single cell information from genomes [59, 60], transcription [54, 69] and protein expression [82], methods for the multi-parametric analysis of metabolites in single cells are scarce [137]. What is more, the great majority of these techniques was developed for mammalian cells.
Some methods with single-cell sensitivity have successfully been transferred to microbes, including genome analysis [186-188] and the characterization of phenotypes using fluorescence [189, 190], Raman microscopy [191] or elemental analysis of isotopes using secondary ion mass spectrometry [192]. However, in general, applying single-cell methods to microbes is challenging due to the great diversity of cell sizes, morphologies and structural properties of cell walls. Therefore, the study of phenotypic variation in microbial systems and its underlying mechanisms is still compromised by technical limitations in microbial systems [7, 40]. We recently developed a method for the label-free analysis of small molecules in single cells of the microalga *Chlamydomonas reinhardtii*, which combines matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) with high-throughput microarray sample-preparation technology. This technique, here called single-cell mass spectrometry (SC-MS), showed the reproducible relative quantitative measurements of more than 15 lipids and pigments in thousands of single cells of a unicellular organism for the very first time [142]. *C. reinhardtii* is a popular model organism in laboratory evolution experiments owing to the tools developed for transformation and sequencing of the genome [193]. Abiotic stress in *C. reinhardtii* is often linked with light availability, or macronutrient limitation. Phosphorous and nitrogen are among the most limiting macronutrients [194]. *C. reinhardtii* lacks the ability to fix nitrogen, however it can use a variety of different nitrogen sources [103]. Metabolic adaptations to nitrogen limitation are well characterized in *C. reinhardtii* and are the subject of continued study [109]. The main metabolic adaptations to nitrogen limitation include the downregulation of photosynthetic activity [107, 108], the accumulation of lipid droplets and the increased mobilization and allocation of internal and external nitrogen [104]. The downregulation of photosynthesis and the accumulation of neutral lipids are attributed to a decreased utilization of reductant energy due to a slowdown in anabolic metabolism [107]. Different strains of *C. reinhardtii* are known to display marked phenotypic differences in their responses to N-
limitation [101]. In a recent study, Malcom and coworkers investigated the genetic basis of growth rate variation across 18 strains of *C. reinhardtii* across 30 environments and found that, while genetically-based phenotypic variation was constrained to only three main axes of selection (types of environmental variation), the response to nitrogen availability was one of them [195].

Here, we extended our SC-MS method to measure the impact of genetic diversity and plasticity (here defined as all phenotypic variation expressed by isoclonal lineages including phenotypic heterogeneity [40]) in generating phenotypic variation under nitrogen-limited conditions. SC-MS was able to detect 26 small molecules from different pigment and lipid classes in individual cells, including chlorophylls, thylakoid lipids, membrane lipids and storage lipids (Table 3). The average lipid composition of *C. reinhardtii* is known to undergo drastic changes upon removal of nitrogen from the culture medium [109]. Here we measured the lipid composition of thousands of single cells to determine the variance and the composition of phenotypes present in different populations over time. We further investigated the correspondence between measures of population-level growth and resource limitation and single-cell phenotypes to investigate the possibility of different strategies in dealing with nitrogen limitation. To do so, we cultured the genetically diverse strain CC-1690 and two isoclonal lineages isolated from CC-1690 termed ANC3 and ANC5 under N-replete and N-deplete conditions for 9 days. Population level characterization showed differences among the lineages selected for this experiment in their population-level growth rate ($\mu_{\text{max}}$, see Appendix 8.2, Figure S 5) and in their minimum nitrogen requirements (referred to here as $R^*$, see Appendix 8.2 Figure S 5). By sampling multiple time points of batch growth for SC-MS, we captured the adaptation to nitrogen depletion in the different cultures with single-cell resolution.
Table 3 Functional assignment of compounds targeted by single cell mass spectrometry and the reported population level responses in literature. The number of carbons and double bonds of lipids are indicated in parentheses, e.g. TAG (52:6). The detected TAG peaks can represent multiple isomers, e.g. TAG (18:0/18:2/16:4), TAG (18:0/18:4/16:2) and other isomers all contribute to the peak at TAG (52:6).

<table>
<thead>
<tr>
<th>Compound class and targeted peaks</th>
<th>Functional assignment</th>
<th>Change under N-limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosynthetic pigments</td>
<td>Light absorbance in photosynthetic complexes.</td>
<td>decrease</td>
</tr>
<tr>
<td>Chlorophyll a, Chlorophyll b</td>
<td></td>
<td>References: [104, 108]</td>
</tr>
<tr>
<td>Thylakoid glycolipids</td>
<td>Constituents of the thylakoid membrane embedding photosynthetic complexes</td>
<td>decrease</td>
</tr>
<tr>
<td>MGDG (34:7), DGDG (34:6), DGDG (34:5), DGDG (34:3), DGDG (34:2)</td>
<td></td>
<td>References: [106, 196]</td>
</tr>
<tr>
<td>Extraplastidic membrane lipids</td>
<td>Extraplastidic membrane lipid that functionally replaces phosphatidylcholine. Present e.g. in endoplasmic reticulum, Golgi or mitochondria.</td>
<td>Increase</td>
</tr>
<tr>
<td>DGTS (34:4), DGTS (34:3), DGTS (34:2), DGTS (36:7), DGTS (36:6), DGTS (36:5), DGTS (36:4)</td>
<td></td>
<td>Reference: [197]</td>
</tr>
<tr>
<td>Neutral storage lipids</td>
<td>Energy storage in the form of reduced carbon. Accumulating in the form of lipid droplets in the cytoplasm.</td>
<td>increase</td>
</tr>
</tbody>
</table>
6.2 Materials and methods

6.2.1 Microbial strains and culture conditions

CC-1690 wild type mt+ was obtained from the Chlamydomonas Resource Center, University of Minnesota (http://www.chlamycollection.org). The culture inoculum from the Chlamydomonas Resource Center was grown up in sterile COMBO freshwater medium [200] lacking silicate, animal trace elements and vitamins, and then again plated onto YA agar plates in order to allow for the formation of individual clonal colonies. Five clonal isolates were selected from agar plates by haphazardly picking five spatially isolated and distinguishable colonies and inoculating them again into liquid medium. These clonal isolates were designated as Ancestors 1-5 (ANC1-ANC5). A detailed description for the determination of population level estimates of growth rates and nitrogen requirements can be found in the Appendix, Chapter 8.2.3.1)

6.2.2 Nitrate limitation experiments

The wild type strain CC-1690 and the two isoclonal isolates, ANC3 and ANC5, were again first acclimated in replete (1000 μM NO₃⁻) and deplete (20 μM NO₃⁻) nitrogen conditions. For each acclimation batch culture, 1 mL of liquid batch culture from replete medium was inoculated into 500 mL of either NO₃⁻ replete or deplete medium, with each population x NO₃⁻ level replicated twice. These batch cultures were allowed to grow at 20 °C, at a light intensity of ca. 100 μEinstein m⁻² s⁻², and under 100 rpm shaking for one week. After one week of acclimation, the cultures were centrifuged at 4,500 rpm for 5 minutes, and the supernatant was decanted. Pelleted algae were then re-suspended in fresh, sterile medium of the corresponding NO₃⁻ concentration to achieve an approximately equal optical density (OD) of 2.6 across populations and NO₃⁻ concentrations. This acclimated and density-controlled culture inoculum was then used to inoculate the single-cell phenotype experiment. We pipetted 1 mL of each inoculum
into 500 mL of either nitrogen replete or deplete sterile COMBO medium. The flasks were again maintained at 20 °C, at a light intensity of ca. 100 µEinsteins m\(^{-2}\) s\(^{-2}\), and under 100 rpm continuous shaking for nine days. For SC-MS, the cultures were sampled 2, 4, 6 and 9 days after inoculation. Samples for estimation of dissolved nitrogen and phosphorus were taken on days 2 and 9 (Further details see Chapter 8.2.3.2). For chlorophyll microscopy we sampled the cultures on days 2 and 9. For the measurement of chlorophyll-a autofluorescence, we removed 1 mL samples and fixed the cells using 10 µL of a glutaraldehyde fixative solution (final fixed concentration is 0.01% paraformaldehyde and 0.1 % glutaraldehyde). Fixed samples were stored at 4°C in amber Eppendorf tubes until analyzed (<3 months). Further details on the measurements and image processing can be found in the Appendix, Chapter 8.2.3.3.

6.2.3 Single-cell mass spectrometry

Single-cell MALDI mass spectrometry was performed as described recently [142]. In short, slides were prepared for SC-MS as follows: on each day each culture covered a complete stainless steel slide (of 1430 spots each). Each spot was 300 µm in diameter and had a center-to-center distance of 720 µm. A layer of matrix, corresponding to approx. 5nl of a 10mg/ml solution of 2, 5-DHB (Sigma Aldrich, 85707) in 80% aqueous acetone, was deposited before spotting the cells. Cells were centrifuged three times at 5'000 g for 5 min and re-suspended in deionized water. The integrity of the cells was monitored by fluorescence microscopy throughout the sample preparation as shown in previously published work [142]. To adjust the number of cells per spot for increased single cell yield on the target the optimal number of drops was determined for each culture using a dilution series (4x4 array of 1, 2, 3, 5, 10 drops of cell solution). The rest of the slide was covered with cells and the cells were quenched immediately using liquid nitrogen. The slide was reconstituted to room temperature in a desiccator and lipids were extracted from the cells and co-crystallized with the MALDI matrix. The extraction was performed in five
consecutive cycles (each approx. 5 nl of 10 mg/ml DHB in 80% aqueous acetone) for homogeneous lipid extraction. The whole procedure was monitored using a confocal fluorescence scanner (LS400, Tecan, Switzerland). The number of cells per spot was determined based on a confocal scan of the slides after quenching with liquid nitrogen. MALDI-MS parameters for the measurements on the MALDI TOF-TOF mass spectrometer (AB Sciex 5800, Sciex, Canada) were as follows: the laser was operated at a repetition rate of 400 Hz and an intensity of 4300-4400 arbitrary units. Spectra were measured for the range of 550-1000 m/z with an extraction delay time of 200 ns, exhaustively ablating each spot using a spiral pattern. More MALDI-MS parameters can be found in [142]. Due to technical problems during the spotting only very few single cells of ANC5 culture were successfully measured on days 4 and 6.

6.2.4 Spectral processing of MS data

Spectra were calibrated internally by using matrix peaks and smoothed using a Savitzky-Golay function using the TOF Series Explorer software (Sciex, Canada). The resulting spectra show a mass accuracy of < 0.03 Da (≤30ppm for m/z=1000 and ≤60ppm for m/z = 500, see Appendix Chapter 8.2.2, Figure S 8) and a resolution of ≈12000 (m/z = 750, see Appendix Chapter 8.2.2, Figure S 9). Peaks were picked using a signal-to-noise > 10 criterion. The spectra were exported as ascii files and peak lists using the Data Explorer software (Sciex, Toronto, Canada). Peaklists holding all peaks with S/N > 10 in the recorded spectra were imported to MATLAB (R2015b). The peak list of each cell was searched for a list of known peaks for which the parameters (measured m/z, peak area, signal/noise and resolution) were imported into MATLAB (version 2015b). The list of targeted peaks and their assignment (see also Appendix, Chapter 8.2.3.4) can be found in the Appendix Chapter 8.2.1, Table S 4. A heatmap of the all the signal-to-noise-ratios of the detected peaks in all single-cell spectra (msheatmap function) collected during the experiment can be seen in Figure S 10 of the appendix, Chapter 8.2.2.
6.2 Materials and methods

6.2.5 Data analysis for single cell mass spectrometry

For multivariate analysis principal component analysis (PCA) was performed in R (3.2.4 Revised) using the princomp-function. A detailed procedure for the handling of the data (including spectral processing), submitted to PCA analysis can be found in the Appendix, Chapter 8.2.2, Figure S 11. The peak areas of all the targeted peaks detected (S/N>10, Limit of detection area = 200) in the single cell mass spectra were imported into R. To exclude random detection only MS that contained at least two detected peaks were included in the multivariate analysis. The recorded peak areas span three orders of magnitude thus the dataset was square root transformed and scaled. A principal component analysis on the dataset, including all lineages, all nitrogen conditions and all days of the time series, was performed. We used ggplot2 function in R including the contour plot tool density2d for visualization of the PCA [201] (see Figure 15). The first five principal components (PC) explain 90.8 % of the observed variance in the overall dataset (see Appendix, Chapter 8.2.2, Figure S 12). The first principal component (49.5 % of variance explained) shows all-negative signs. The fact that all PC loadings have the same sign means that this PC does not refer to relative but to absolute intensities, which is underlined by the fact that the loadings closely resemble the average intensity of the peak in the experiment (see Appendix, Chapter 8.2.2, Figure S 13). In MALDI mass spectra, only relative abundances hold quantitative information thus we restricted our interpretation on the other principal components showing loadings with opposite signs. This is especially relevant in single-cell analysis, since there is no option for introducing an internal standard and there is no single compound that is present in equal amounts in each cell. The second principal component explains 26 % of the observed variance and segregates between photosynthetic pigments and the extraplastidic DGTS lipids (see appendix, chapter 8.2.2, Figure S 13). Principal component three explains 8.17 % of the observed variance and segregates cells that detect high levels of TAG storage lipids. Principal component 4 explains 4.2 % of the observed
variance and segregates between DGTS lipids of different fatty acid composition.

The principal component scores for PC2 and PC3 of each population were tested for normality using the Shapiro-Wilk's test swtest.m in MATLAB [107]. Only 5 out of 24 measurements were normally distributed in both PC2 and PC3 (p=0.05). Significant differences among samples were detected using the nonparametric Mann-Whitney-U-test using ranksum function in MATLAB. Differences in variance were analyzed using the non-parametric Conover's-Squared-Ranks test for equal variances by Conover [202, 203]. Hypothesis testing was Bonferroni corrected to give significance levels higher than 0.01. The reason for choosing such a conservative significance level for testing was the number of parameters included in the PCA and the high differences in spectral intensity between different cells measured.

Based on plots of cellular phenotypes on PC2 and PC3, we assigned cells to one of three apparent phenotypic categories. Based on the cut-offs of the PC scores, we defined three categorical phenotypes as ‘high chlorophyll’ (PC3 < 5, PC2 > 0), ‘high membrane lipid’ (PC3 < 5, PC2 < 0) and ‘high storage lipid’ (PC3 > 5). The absolute numbers of cells in each phenotypic class can be found in the Appendix, Chapter 8.2.1, Table S 5. Contingency table analysis of Table S 5 by means of the Fisher’s exact test was performed using Myfisher23.m function [204]. Details on the bivariate data analysis can be found in the Appendix, Chapter 8.2.3.5.
6.2 Materials and methods

Figure 15 Contour plots of the PCA performed on the single-cell mass spectra. PC2 segregates between ‘high chlorophyll’ and ‘high membrane lipid’ cells corresponding to high and low PC2 values respectively. PC3 additionally segregates ‘high storage lipid’ cells. There is a general trend of all N-depleted populations from ‘high chlorophyll’ via ‘high membrane lipid’ towards ‘high storage lipid’.
6.3 Results

6.3.1 Single cell mass spectrometry

SC-MS values report differences in relative lipid and pigment composition of the single cells. The heat maps of the targeted peaks detected in the single cells of different cultures on different days can be found in the Appendix, Chapter 8.2.2, Figure S 10.

To disclose important phenotypic differences among the three lineages, CC-1690, ANC3 and ANC5 in response to nitrogen depletion treatment we used principal components analysis to decompose the variation in the mass spectra. PC1 refers to the absolute intensities of peaks in the mass spectra that cannot be assessed quantitatively using our MALDI-MS method (see Methods section). PC2, however, separated between chlorophylls and DGTS while PC3 additionally separated between TAGs (Figure 15, for the PC loadings see the Appendix, Chapter 8.2.2, Figure S 13). Based on this analysis, we defined three phenotypes called ‘high chlorophyll’ (PC2 > 0 and PC3 < 5), ‘high membrane lipid’ (PC2 > 0 and PC3 <5) and ‘high storage lipid’ (PC3 > 5) (see Appendix, Chapter 8.2.1, Table S 5). For exemplary spectra of the three phenotypes, see Figure 16B. For PC scores of the respective cells see the Appendix, Chapter 8.2.2, Figure S 14. The ‘high chlorophyll’ phenotype was ubiquitous in N-replete cultures and especially prominent on day 2 (e.g. 97.4 % of cells measured in N-replete CC-1690). Fisher's exact test showed that in deplete cultures there was no significant difference between the phenotype frequencies in CC-1690 and ANC3 on day 2 (p=0.20) while CC-1690 and ANC5 differed significantly (p=0.008). With increasing nitrogen limitation ‘high chlorophyll’ phenotypes made up a decreasing fraction of the population (see green circles in Figure 16A) while the fraction of ‘high membrane lipid’ phenotypes increases (Table 3).
6.3 Results

**Figure 16A** Schematic of the relative phenotypic composition of the populations over time. Each circle represents a 5% fraction of cells within the population (for more details see also Appendix, Chapter 8.2.1, Table S5). Green circles represent the ‘high chlorophyll’, blue circles the ‘high membrane lipid’ and red circles the ‘high storage lipid’ phenotypes.

**16 B** Exemplary single cell mass spectra of the three phenotypes ‘high chlorophyll’, ‘high membrane lipid’ and ‘high storage lipid’. The spectra were background subtracted using a proximate empty spot mass spectrum. ‘High chlorophyll’ and ‘high membrane lipid’ cells are from replete ANC3 culture on day 9. ‘High storage lipid’ cell is from deplete ANC3 culture on day 9.
Focusing on the most abundant chlorophyll (chlorophyll a) and the most abundant extraplastidic membrane lipid in the spectra (DGTS (34:3)) a bivariate analysis revealed a marked transition between ‘high chlorophyll’ stages on day 2 and ‘high membrane lipid’ on day 9 in N-deplete cultures as can be seen in Figure 17. Remarkably, CC-1690 shows a distinct bimodal distribution including both spectra high in chlorophyll a and high in DGTS (34:3) on day 9. The three lineages also differed in the timing of the onset of the ‘high storage lipid’ phenotypes (Table 3) in N-depleted cultures (Figure 15 and Figure 16A). In CC-1690 we detected ‘high storage lipid’ phenotypes throughout the time series (12.5 – 36.3 % of the cells in the population) while in ANC3, only small fractions of the populations showed ‘high storage lipid’ phenotype until day 6 (1.6% on day 2, 4.8% on day 6, see also Appendix, Chapter 8.2.1, Table S 5). ANC5 showed a very small fraction of ‘high storage lipid’ phenotypes on day 4 (3.9%) and on day 6 (0.6%). Highest frequencies of ‘high storage lipid’ phenotypes for both isoclonal lineages were found on day 9. ANC3 contained almost 40% of cells with a ‘high storage lipid’ phenotype while ANC5 contained the lowest level of ‘high storage lipid’ phenotype cells (14.8%) in comparison to the other two lineages. Fisher’s exact test showed that under deplete conditions, CC-1690 differed significantly both from ANC3 (p = 0.00008) and from ANC5 (p=0) on day 9. In CC-1690, phenotype frequencies were not significantly different between day 6 and day 9 (p=0.91). CC-1690 was the first culture to respond to nitrogen stress with ‘high storage lipid’ phenotypes, but also the population that was able to retain the highest fraction of ‘high chlorophyll’ cells until day 9 (15.6 % in CC-1690, 3.6% ANC3 and 3.2% in ANC5). Furthermore, CC-1690 N-deplete and N-replete cultures displayed greater phenotypic similarity on day 9 than ANC3 and ANC5, as shown by the high overlap of contour plots of CC-1690 N-replete and N-deplete cultures in Figure 15.

6.3.2 Analysis of variance within populations

The variance in PCA scores were used as indicators of phenotypic variance within each population, in order to examine, if the
whole range of phenotypes caused by plasticity in the isoclonal lineages is also present in the genetically diverse population. Therefore, we tested if variance in PC3 scores of N-depleted CC-1690 cultures was higher on each of the individual days. CC-1690 showed significantly higher variance in PC3 on day 4 and day 6 compared to ANC3 and ANC5 (Squared ranks test, p=0.01). On day 9 distributions of CC-1690 and ANC3 were not significantly different (U-test, p=0.26). Both of their variances were, however, significantly higher than variance of ANC5 (Squared ranks test, p=0.01).

Furthermore, we wanted to test if variance in the PC2 and PC3 scores were different between deplete and replete environments. As expected based on the higher abundance of TAG in N-deplete environments, in 7 of 9 cases tested PC3 variance was higher in deplete populations (Squared ranks test, p = 0.01, measurements n<10 were excluded) and never higher in replete populations. However, in PC2 the variance was significantly higher in N-deplete populations in 2 cases, whilst in 4 cases, N-replete populations were significantly higher in variance (p=0.01).

### 6.3.3 Microscopy and population level measurements

The two isoclonal lineages had been selected for the SC-MS experiment based on their different population-level behavior under N-deplete conditions compared to their parental strain CC-1690 (Appendix, Chapter 8.2.2, Figure S 5 – S 7). ANC3 showed a high growth rate ($\mu_{\text{max, ANC3}} = 0.96 \text{ d}^{-1}$) and a higher nitrogen requirement ($R^*_{\text{ANC3}} = 1.78 \text{ µM N}$) than CC-1690. ANC5 showed a lower growth rate ($\mu_{\text{max, ANC5}} = 0.54 \text{ d}^{-1}$) than CC-1690 and a nitrogen requirement of $R^*_{\text{ANC5}} = 0.5 \text{ µM N}$. The genetically diverse population CC-1690 had a growth rate of $\mu_{\text{max, CC-1690}} = 0.80 \text{ d}^{-1}$ and a low nitrogen requirement ($R^*_{\text{CC-1690}} = 0.27 \text{ µM N}$). Since SC-MS only reports relative compound levels and we cannot even assume one of the compounds to be present in equal amounts in all the single-cells measured, we determined chlorophyll levels using fluorescence microscopy to see how
chlorophyll levels of different lineages compare within the time-course of the experiment.

On day 2, the N-replete and N-deplete cultures showed similar chlorophyll levels while on day 9 replete cultures were much higher in chlorophyll (Figure 18). Fluorescence microscopy suggests that ANC5 cells have very low absolute levels of chlorophyll on day 9. ANC3 still shows the highest absolute chlorophyll intensities of all lineages under N-deplete conditions on day 9 (Figure 18).

**Figure 17** Violin plots of the log-transformed ratio of the peak area of DGTS (34:4) and chlorophyll a. Histograms are smoothed using a kernel density estimation. Red crossed indicate population averages. Replete ANC5 cultures on day 4 and day 6 showed too few successful measurements (less than 10) to be included in the analysis.
Figure 18 Chlorophyll fluorescence levels in relation to cell size determined using fluorescence microscopy.
6.4 Discussion

The single-cell phenotype data collected here is the first of its kind in term of complexity and extent. The depth of information collected from each cell revealed the marked cell-to-cell variation within each culture at each time point. On population average, the measurements follow the general trends reported in the literature, including the decrease in chlorophyll, an increase in DGTS content or the accumulation of TAG’s [108, 109, 197]. Following the phenotypic compositions of the populations at the single-cell level over time renders a dynamic picture of phenotypic variation in adaptation.

Our results show that genetic diversity manifests in greater phenotypic variation among cells within a population and in their heterogeneous susceptibility to N-limitation (Figure 16A). Phenotypic variation (‘high chlorophyll’, ‘high membrane lipid’ and ‘high storage lipid’) in the genetically diverse population is maintained over the time course of the experiment, while the isoclonal populations show more synchronicity in their response to N-limitation; both of these observations suggest a more homogeneous susceptibility towards this stress. It has been reported that the accumulation of high levels of TAG are a mechanism to alleviate oxidative stress concurrent with the slowdown of anabolic metabolism upon N-limitation [107]. We therefore attribute the ‘high storage lipid’ phenotype to cells that experience high N-limitation stress. While both isoclonal lineages showed ‘high storage lipid’ phenotypes almost exclusively on day 9, the diverse population already showed cells with ‘high storage lipid’ phenotype within the first 6 days of the experiment. By contrast, CC-1690 also included the highest fraction of cells with a ‘high chlorophyll’ phenotype on day 9. The ‘high chlorophyll’ phenotype has a high frequency in the replete cultures, especially on day 2, at which point it makes up more than 95% of the detected cells in CC-1690. The presence of ‘high chlorophyll’ cells on day 9 might be an indication of cells that are little affected by the N-limitation. This could provide an explanation why the population level nitrogen requirement R* is lower in CC-1690
than in either of the two isoclonal lineages, while maintaining a high maximum specific growth rate (Appendix, Chapter 8.2.2, Figure S 5 and Figure S 6). Alternatively the ‘high chlorophyll phenotypes’ in CC-1690 on the last day of the experiment might also result from heterogeneous microenvironments or constitute a form of non-adaptive phenotypic variation [179]. The functional classification of the ‘high membrane lipid’ phenotype is more difficult because extraplastidic membranes are involved in a variety of metabolic processes in the ER, the Golgi apparatus and the plasma membrane (Table 3). Despite the clear change in DGTS/chlorophyll a ratio in N-deplete cultures (Figure 17), the abundance of the ‘high membrane lipid’ phenotype and the variability of DGTS/chlorophyll a ratio in replete cultures suggests that the increase of ‘high membrane lipid’ phenotypes is not only a response to N-limitation, but is also part of the phenotypic variation in replete environments. Especially since the change in the ratio of the two peaks in SC-MS could result from either the down regulation of chlorophyll or the up-regulation of DGTS membrane lipids.

SC-MS suggested that ANC3 underwent a transition from ‘high chlorophyll’ to ‘high membrane lipid’ phenotypes early on (70% ‘high membrane lipid’, 30% ‘high chlorophyll’ on day 4). Fluorescence microscopy of the same population revealed that ANC3 still shows highest chlorophyll levels on day 9 compared to CC-1690 and ANC5 (Figure 18). This suggests an up-regulation of DGTS and that downregulation of photosynthesis might be less pronounced in ANC3, which corresponds well with the high fraction of ‘high storage lipid’ phenotypes in ANC3 on day 9. This is also in agreement with the significantly higher nitrogen requirement R* of ANC3 compared to CC-1690 (Appendix, Chapter 8.2.2, Figure S 6). ANC5, on the other hand, showed lowest chlorophyll values per cell compared to both CC-1690 and ANC3 on day 9 in fluorescence microscopy. SC-MS measurements of N-deplete ANC5 showed a decrease in the chlorophyll/extraplastidic membrane lipid ratio on day 4-9. However, the fewer ‘high storage lipid’ cells (14.8%) compared to both ANC3 and CC-1690 suggest an adaptive downregulation of photosynthesis. Population measures showed that
ANC5 has a significantly lower population-level growth rate, which further supports the hypothesis that ANC5 shows conservative growth behavior beneficial in nutrient limited environments. Based on these responses of the isoclonal lineages, it would stand to reason that they represent different strategies in coping with nutrient limitation. By keeping photosynthetic activity low, ANC5 decreases its risk of running into oxidative stress upon intensification of nitrogen limitation. On the other hand, in keeping high amounts of chlorophyll, ANC3 might utilize available nitrogen more quickly (see Figure 18 but also Appendix, Chapter 8.2.2, Figure S 6 and Figure S 7).

Timescales of environmental fluctuations are of great importance to the responsive strategies of organisms [205, 206]. Therefore, different strategies can be beneficial in the face of different conditions of nutrient stress, in terms of both the severity and timescales of stress. ANC5 might be an evolutionarily stable strategy in an environment that undergoes long periods of starvation, while ANC3 might prevail under high N-conditions. Extrapolating the phenotypic differences in our randomly picked clonal isolates, the genetically diverse CC-1690 population would therefore be expected to hold genotypes with many different strategies, which would allow the population to better cope under fluctuating environments. Different strains of *C. reinhardtii* show marked differences in their resilience to N-limitation and accumulation of storage lipids [101, 195]. Therefore, the genetic background is the most likely source of phenotypic variation and the different observed isoclonal strategies in response to environmental change.

Multi-parametric phenotype data from single cells, such as those presented here, are invaluable in the study of phenotypic adaptation. The key abilities of SC-MS are that (i) multiple cellular processes can be monitored simultaneously, (ii) high-throughput analysis can render a cross-section of the phenotypic composition of populations, and (iii) the method can be applied on multiple time points in an experiment, which reveals information on changes in the phenotypic composition of populations over time. Our experiments also show the benefit of
complementing single cell mass spectrometric data with other phenotypic data collected from the same population and the bridging of single-cell information with population characteristics, which enhances this highly multiparametric experimental approach. The experiments showed here further underline that SC-MS can detect biologically functional phenotypic variation in populations, i.e. differences between cells that can make a difference for the whole population.
7 Conclusions and Outlook

The implementation of microarray sample preparation technology has enabled the high throughput in sample preparation that so far constituted the critical bottleneck for the application of MALDI-MS for the analysis of single cells. Microarraying technology has not only allowed to increase throughput but also added precision and reproducibility that previous protocols for using MALDI as an ionization method in the analysis of single cells lacked. Therefore the method now allows to take full advantage of MALDI-TOF-MS which include the low dilution factor and the speed of spectral acquisition which give MALDI a clear advantage over methods employing electrospray ionization [207]. Therefore the method now allows to acquire thousands of spectra from single cells within a day of experiments. Using DHB as a matrix in positive ion mode the list of molecules that can be targeted includes more than 30 pigment and lipids. The application of the method for the measurement of variation of lipid composition within populations of nitrogen limited cells has generated biological insights on the genetic impact for the observed phenotypic variation.

The successful application of the method and the biological insights generated raise a number of questions concerning future applications.

1. Bioanalytical questions
   a. To what extent can the method be transferred to other organism, e.g. bacteria, non-fluorescent species?
   b. What other compound classes could be analyzed?
   c. Can we introduce an internal standard for single-cell MALDI-MS measurements?

2. Biological questions.
   a. What is the impact of selection on the phenotypic composition of a genetically diverse population?

These issues will be addressed and discussed in the following paragraphs of this final chapter.
7.1 Bioanalytical Questions

7.1.1 Transferability to other systems

There are several unresolved questions concerning the applicability of the method to other biological systems. The use of autofluorescence for the detection of single-cell spots on the stainless steel MALDI target at first sight limits the method to unicellular organisms with autofluorescent pigments. On the other hand, fluorescence could easily be introduced to cellular systems, e.g., by transfection with fluorescently labelled proteins or by staining with fluorescent dyes. It should be mentioned here that many popular small fluorescence dyes carry charges or are easily charged, which improves their solubility in aqueous systems, e.g. cyanine dyes, DAPI, or fluorescein. Furthermore, they often have high extinction coefficients in the UV range. Thus these molecules will also ionize in the MALDI process and are potential suppressors of cell-derived signals. Thus when considering small dyes compatible with MALDI one should take these properties into account.

Another question concerns sensitivity. That question is more difficult to address. The most important issue in the development of a single cell method in *Chlamydomonas reinhardtii* was efficient cell lysis. It is that crucial step in sample preparation that makes all the difference between an excellent spectrum and no detected peaks at all (Figure 7). Thus only after optimization of cell lysis a statement can be made on the detectability of any compound in any type of single cell. We have taken several attempts at measuring single bacteria. We started out at measuring *Thiodictyon synchronous* in normal stainless steel MALDI slides of 300 µm spot diameters. One major problem in the case of detecting small bacteria is the resolution of the confocal laser scanner used to detect the cells on the chip, i.e. we were not sure how many cells were exactly in the spots. Bacteriochlorophyll peaks showed up during spectral acquisition but not in the resulting spectra as the peaks were only present in very few of the acquired laser shot positions. As a consequence of this we asked our collaborators to produce a smaller
chip with 100 µm spot diameters. This chip was used to measure both *Synechococcus* and *Thiodictyon* synchronous (an isolated purple sulfur bacterium from lake Cadagno) in which we were able to detect bacteriochlorophylls without the certainty that it was actually a single bacterial cell. Whether there is indeed single cell sensitivity and whether other compounds can be detected from these organisms still has to be verified.

### 7.1.2 Compound classes

MALDI mass spectrometry is highly successful in lipid profiling as can be seen from MALDI imaging experiments. There are several factors contributing to this success. First many of the lipids detected are structural components of the cell and are therefore abundant. Furthermore as a consequence of their lipophilicity they have to be extracted and co-crystallized with the MALDI matrix using non-polar solvents. Since other ions which often have suppressive effects on ion formation are highly hydrophilic, this procedure is equivalent to a liquid extraction and purifies the lipids to be analyzed. This is especially relevant in biological samples like tissues or cells where there is little to no sample pretreatment such as desalting.

Seemingly contradictory to this, using negative ion mode and 9-aminoacridine for single cells MALDI-MS enables the detection of highly polar small biomolecules such as nucleotides. Spectra recorded from single cells with 9-aminoacridine show a striking prevalence of molecules containing phosphate or sulfate groups. These discrepancies between different matrices underline the importance to investigate the mechanistic aspects of MALDI ionization to enable the design of new matrices.

### 7.1.3 In search of an “internal” standard

The application of an internal standard is a frequently discussed issue in MALDI mass spectrometry [208]. The difficulty of homogeneous crystallization and extraction have led to heated debates at conferences on quality standards especially for absolute quantitation
of compounds. The main issue is that when it comes down to single cells there is no single compound detectable by mass spectrometry that stays constant from one cell to the next. Thus spiking a substance to serve as internal standard would be an alternative. However since especially for microbial samples the extraction of analytes from the cells is a huge challenge the introduction of an internal standard would comprise a similar challenge. Despite the large amount of information generated, it is highly recommended to use single cell mass spectrometry not as a sole methodological approach in the study of biological systems but rather in in combination with other measurements. For example, as described in Chapter 6 the ability to detect chlorophyll by mass spectrometry was complemented by fluorescence detection in and a variety of population measurements.

7.2 Biological Questions

7.2.1 Impact of natural selection

The SC-MS analysis of genetically diverse populations revealed a significant contribution of genetic variation to the overall observed phenotypic variation (see Chapter 6). However, due the lack of a single-cell whole genome sequencing protocol, we were not able to estimate the impact of selection, i.e. changes in allele frequencies, on the observed changes in phenotype composition. This could be especially relevant, since asexual reproduction and the absence of nitrogen limitation in the recent history of the strain CC-1690 could result in a selective sweep in the population, as it is confronted with a nitrogen limited environment [21]. An ideal workflow for the study of laboratory evolution would couple single-cell phenotyping and genotyping, ideally measured in the same cell, and could thereby directly link the two levels. The compatibility of the cell lysis employed for single-cells with genomic sequencing would therefore supply an immense boost of the SC-MS workflow in the study of laboratory evolution.
8 Appendix

8.1 Appendix to Chapter 4

The here contained supplementary material refers to Chapter 4 and was also published online:

http://aem.asm.org/content/81/16/5546/suppl/DCSupplemental
8.1 Appendix to Chapter 4

8.1.1 Supplementary Tables

Table S 1 Sample preparation protocol for high-throughput single-cell measurements

Protocol for the preparation of microbial cells for a single cell experiment using MALDI microarray plates.

1) The empty microarray slide is scanned to check for auto-fluorescent dust.
2) 10 drops (4-5 nanoliter) of MALDI-matrix solution (for DHB 10mg/ml in 80 % acetone 30 % water) are spotted into each well of the array.
3) The cells are washed with deionized water prior to the spotting. For this purpose cells are centrifuged three times (2000 x g for 5 min in the case of Chlamydomonas reinhardtii) and re-suspended in water (HPLC grade) to remove salts and lysate that might suppress or bias signals measured in single-cell MALDI-MS.
4) A dilution series of cells is spotted to determine the number of drops resulting in the maximum number of wells, containing single cells. A concentration of around 10^6 cells per ml should result in an average of 0.5 cells per drop (≈0.5 nl). By testing the results for 1,2,3,5 or 10 drops one can account for effects like clustering or cell divisions. The Fluorescence scan reveals the number of drops for maximal single cell yield.
5) Cells are then spotted onto the whole array and then subjected to a fluorescence scan.
6) Cells are then lysed and their metabolism quenched by submerging the slide in liquid nitrogen. The cell walls are cracked by the immediate crystallization of water inside the cells. The slide is reconstituted to room temperature in a desiccator to avoid condensation on the slide that could reposition cells or lead to cross-contamination between
wells. To check for cell displacements, the slide can be subjected to another fluorescence scan.

7) Then MALDI matrix is applied onto the cracked cells to extract analytes from the cells and co-crystallize them with the MALDI-matrix. For *Chlamydomonas reinhardtii* we found that 50 drops (20-25 nanoliter) applied in 5 consecutive spotting runs of 10 drops worked best. Applying 10 drops of matrix consecutively means that analytes are extracted in five consecutive extractions after each of which matrix crystallizes with the extracted analytes, which makes this step more robust and reproducible. To monitor the extraction process the slide is subjected to another fluorescence scan.

8) Cells are then put into plastic tubes and stored under a nitrogen atmosphere at -80° C until measured with MALDI-MS.
### Table S 2 Compounds detected in a single *Chlamydomonas reinhardtii* cell using MALDI-FT-ICR-MS

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Formula</th>
<th>Measured m/z</th>
<th>Theoretical m/z</th>
<th>δ (ppm)</th>
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<td>Thylakoid lipids</td>
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<td>767.47047</td>
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8.1.2 Supplementary Figures

Figure S 1 MS/MS spectra of the different lipid classes detected in *C. reinhardtii*. 
**Figure S 2** UV-VIS spectra of *C. reinhardtii* cell cultures.

**Figure S 3** Chlorophylls detected from single cells. The wild-type strain shows both chlorophyll a and chlorophyll b while the chlorophyllide a oxidase mutant shows only chlorophyll a and no chlorophyll b. Thus the two strains can be differentiated based on the presence or absence of chlorophyll b.
Figure S 4 Effect of incomplete cell lysis on the depletion of chlorophyll fluorescence by the MALDI ablation process. A Single cells are placed into microarray spots. B Chlorophyll that stays inside the cell is not co-crystallized with matrix and therefore ionized much less efficiently. Efficient lysis is therefore a prerequisite for reproducible and good quality mass spectra. C Chlorophyll that is not extracted stays inside the cells despite the laser ablation.
The here contained supplementary material refers to Chapter 6, work submitted to ISME Journal: Krismer, J., Tamminen, M., Zenobi, R., Narwani, A. “Single-cell mass spectrometry reveals the importance of genetic diversity and plasticity for phenotypic variation in nitrogen limited Chlamydomonas”
### Supplementary Tables

**Table S 3** Phosphate, total phosphorous and nitrate levels of the media determined on day 2 and 9 of the experiment in the filtered media.

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Table S 4  Compounds and corresponding ionic species detected in mass spectrometry.

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<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (50:3)</td>
<td>867.68</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (50:4)</td>
<td>865.67</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (50:5)</td>
<td>863.65</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (50:6)</td>
<td>861.64</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (50:7)</td>
<td>859.62</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (52:3)</td>
<td>895.72</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (52:4)</td>
<td>893.7</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (52:5)</td>
<td>891.68</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (52:7)</td>
<td>887.65</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (52:8)</td>
<td>885.64</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (52:9)</td>
<td>883.62</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
<tr>
<td>TAG (52:10)</td>
<td>881.61</td>
<td>[M+K(^+)]</td>
<td>Yang et. al 2015, Liu et. al 2013</td>
<td></td>
</tr>
</tbody>
</table>

*Suppressive interfering Chl fragments

* |
Table S5 Contribution of the different phenotypes to the populations at different time points of the experiment including the total number of cells measured. The relative fraction of each phenotype within the population are indicated in percent.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-1690</td>
<td>1.53%</td>
<td>8.5%</td>
<td>0.05%</td>
<td>1.32%</td>
</tr>
<tr>
<td>ANCS depleted</td>
<td>10.1%</td>
<td>1.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>ANCS Replete</td>
<td>9.1%</td>
<td>1.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

Percent
8.2.2 Supplementary Figures

**Figure S 5** Maximal growth rates (µmax) and required nitrogen (R*) of the different strains determined from population growth measurements under different N-regimes.

**Figure S 6** Population growth rates determined under varying nitrogen conditions.

**Figure S 7** Population level growth curves for the selected strains under N-replete (1000 µM Nitrate) and N-deplete (20 µM Nitrate) conditions. Mean and SD of three biological replicates.
Figure S 8 Histogram showing the measured mass of DGTS (34:4) in all the mass spectra collected (theoretical mass is 732.5773 Da). Normal distribution is indicative of the lack of interfering peaks in the spectra that could emerge from contaminating molecules with similar but slightly different m/z.

Figure S 9 Histogram showing the resolution of the peak of DGTS (34:4) in all the spectra recorded. Smooth Gaussian distributions suggest no interfering peaks which would decrease resolution and disturb the Gaussian profile.
Figure S 10 Heat maps of the signal/noise value of the peaks detected in single-cell mass spectrometry. All maps are scaled to the same max. value of S/N 100.
**Figure S 11** Data handling procedure for SC-MS data. Multiple quality control steps were included to reduce the noise in the resulting highly multivariate data set submitted to PCA.
Figure S 12 Cumulative variance explained by principle components of the PCA comprising single-cell measurements of the whole time-series experiment (all strains, all N-conditions, all time points).
Figure S 13 Loadings of the first four principle components (87.93 % of the variance explained). Contributing MS-peaks are colour coded based on compound class (pigments in green, extraplastidic lipids in blue and TAG in red). PC1 contains all negative loadings and accounts for differences in absolute intensities which cannot quantitatively assessed using our SC-MS method.
Figure S 14 Phenotype classification based on principle component scores in PC2 and PC3 and corresponding single cell spectra. Single cell spectra are background corrected by subtracting a nearby spot on the array containing no cell. Lower spectra are flipped for better comparability of the corresponding mass signals.
8.2.3 Supplementary Material and Methods

8.2.3.1 Population-level nitrogen-requirements

In order to determine the minimum nitrogen requirements for positive population-level growth for these five clonal isolates and CC-1690 (six populations), we ran experiments to measure their growth rates as a function of the nitrate (NO$_3^-$) concentration in liquid medium. The gradient included ten nitrate levels: 0, 0.2, 2, 20, 40, 60, 80, 100, 150 and 200 µmol NO$_3^-$ L$^{-1}$ (all other resources were kept constant). We first acclimated each population to each of the ten the NO$_3^-$ concentrations which they would experience during the experiment. The purpose of the acclimation was to ensure that the algae were responding to the ambient nitrogen levels, and were not transporting and using stored nitrogen during experiment. Prior to the acclimation, the algae were centrifuged at 5,000 RPM for 15 minutes at 20°C and rinsed with NO$_3^-$-free media twice. The populations were then diluted by 1 in 100 or 1 in 10, according to their initial densities into 10 mL of sterile media with the specific NO$_3^-$ concentration to which they would later be subjected during the experiment. The aim of the dilutions was to achieve a low enough density that species could acclimate to the new resource condition without experiencing density-dependent growth. Chlorophyll-a fluorescence, measured as raw fluorescence units (RFU) at an excitation wavelength of 435 nm and emission 685 nm, served a proxy for algal population-level biomass. The acclimation period lasted 5-6 days, after which we measured the chlorophyll-a fluorescence of the populations to determine whether particular populations needed to be diluted again before being inoculated into the experimental conditions. We aimed to achieve an inoculation RFU <150 in order to limit density-dependent growth in the initial days of the experiment. Because the inoculation process required a 1 in 10 dilution, we diluted the acclimation cultures again before inoculation if a population exceeded 1500 RFU.

Growth rate experiments were conducted in sterile 48-well tissue culture plates. Inoculations took place over the course of two
consecutive days. Algal populations were inoculated by pipetting 100 µL of acclimated culture into 900 µL sterile NO$_3^-$-controlled medium. The algal populations were randomly allocated into wells across the well-plates, and individual plates had unique NO$_3^-$-concentrations in the media. Plates were covered with Breathe Easy™ permeable membranes to minimize evapotranspiration and the risk of cross-contamination. Each well-plate was randomly placed on a shaking table, which was used to shake the plates for one hour per day before reading them using a Biotek Synergy plate reader. The plates were kept on the shaking table in a culturing room at 20 °C under red and blue LED grow-lights (Phillips GreenPower LED Production modules) at a light intensity of ca. 100 µEinstein m$^{-2}$ s$^{-2}$. The plates were also shaken in the plate reader for 15 seconds before fluorescence measurements were taken. In order to estimate population-level growth rates, chlorophyll-a RFU was measured at the same time daily for nine days. Each population x NO$_3^-$ treatment was replicated three times. With all time-series data in hand, we estimated population-level growth rates by estimating the slope of the linear regression of natural log-transformed RFU over time. We then plotted the growth rates of each population as a function of NO$_3^-$ concentration, having three replicates per level. We then fitted the Monod model to the growth rate data using methods described in[209]. To summarize briefly, the model is:

$$\mu(R) = \frac{\mu_{max}R}{R + \frac{\mu_{max}}{\alpha}} - D$$   \hspace{1cm} (1)$$

Where $\mu(R)$ is the growth rate of a population at a given NO$_3^-$ concentration (R), $\mu_{max}$ is the population maximum growth rate when NO$_3^-$ is unlimited, $\alpha$ is the populations’ initial response to elevated resource availability and D is the dilution or death rate. We used non-linear least squares regression to estimate $\alpha$ and $\mu_{max}$ and their standard error in R. $R^*$ values were obtained by solving the Monod model analytically in Mathematica. Confidence intervals were calculated using the lower and upper limit of the 95 % confidence interval of $\alpha$ and $\mu_{max}$
which were obtained by error propagation using Monte Carlo simulation and Taylor expansion in R.

This analysis of population-level growth under varying nitrogen conditions showed that Ancestors 3 and 5 were the most unique among the clonal isolates (Appendix, Figure S 5). Ancestor 3 had a higher $\mu_{\text{max}}$ than CC-1690, while Ancestor 5 had a lower $\mu_{\text{max}}$ than CC-1690 (Appendix, Figure S 6). Moreover, Ancestor 3 had a higher minimum nitrogen requirement, or $R^*$ for $\text{NO}_3^-$, than CC-1690, whereas the $R^*$ for $\text{NO}_3^-$ for Ancestor 5 was not different from CC-1690 (Appendix, Figure S 5). Time resolved growth further suggests that upon inoculation into fresh medium ANC3 resume growth faster than ANC5 (Appendix, Figure S 7). As a result of these unique population-level traits, we selected Ancestors 3, 5 and CC-1690 for further examination of individual level phenotypic variation within the populations over time in response to nitrogen depletion.

8.2.3.2 Measurements of Nitrogen and Phosphorous content

For these measurements, 15 mL of algal culture was filtered through 0.45 um nitrocellulose filters to remove particulates, and then frozen at -20°C until further within one month. Phosphate and total phosphorous (after an acid digestion in a Systec 2540 EL autoclave) were determined using a photometer (Cary 30 Bio, Varian). Nitrate levels were determined using a “Metrosep A Supp 5” column (100 x 4 mm, Metrohm) for ion chromatography (930 Compact IC Flex, Metrohm). The replete medium contains initially 14 mg N/L, around 10 mg N/L on day 2 and decreases to 2 mg N/L on day 9 of the experiment (Appendix, Table S 3). Considering the nitrogen requirements of the different lineages (Appendix, Figure S 5) nitrogen is thus not a limiting factor for growth in replete cultures.

8.2.3.3 Microscopy

Chlorophyll measurements were performed on a Nikon TI-E microscope equipped with a Perfect Focusing System (Nikon
Instruments) and an Orca-Flash 4.0 camera (Hamamatsu). Pictures were recorded using a 20 x objective (S Plan Fluor ELWD 0.5 NA). Both phase contrast and chlorophyll a (ex. 405 nm, em. 684 nm) channels were recorded in automated fashion using PFS.

The images were processed using CellProfiler [210]. For chlorophyll measurements the cells were detected based on phase contrast outlines (smoothed and declumped based on shape and cell size of 5-20 µm).

### 8.2.3.4 Peaks detected in single-cell mass spectrometry

Assignments of polar lipids and pigments were reported in earlier work by MS/MS from single *C. reinhardtii* cells [211]. Signals previously unknown from single cell measurements including TAG’s were assigned using bulk cell MS/MS (see Appendix, Chapter 8.2.1, Table S 4) and *C. reinhardtii* literature[197, 198]. Due to the lack of easily charged groups, detection limits for neutral lipids like TAG are higher than for polar lipids, e.g. DGTS [212].

### 8.2.3.5 Bivariate Analysis of the data

For the creation of Violin plots for Figure 3, we used the distributionplot.m function. The log transformed ratio of peak areas was plotted as a smoothed histogram using ksdensity where the kernel is robustly estimated via histogram.m normal kernel. In order to account for the profound changes in the spectra during the experiment, i.e. some peaks newly appearing and others completely disappearing we used a fixed LOD (peak area= 200) for the case of non-detection of one of the two peaks analyzed.
9 References


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