

Video Article

High-Throughput Robotically Assisted Isolation of Temperature-sensitive Lethal Mutants in *Chlamydomonas reinhardtii*

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Abstract

Systematic identification and characterization of genetic perturbations have proven useful to decipher gene function and cellular pathways. However, the conventional approaches of permanent gene deletion cannot be applied to essential genes. We have pioneered a unique collection of ~70 temperature-sensitive (ts) lethal mutants for studying cell cycle regulation in the unicellular green algae *Chlamydomonas reinhardtii*¹. These mutations identify essential genes, and the ts alleles can be conditionally inactivated by temperature shift, providing valuable tools to identify and analyze essential functions. Mutant collections are much more valuable if they are close to comprehensive, since scattershot collections can miss important components. However, this requires the efficient collection of a large number of mutants, especially in a wide-target screen. Here, we describe a robotics-based pipeline for generating ts lethal mutants and analyzing their phenotype in *Chlamydomonas*. This technique can be applied to any microorganism that grows on agar. We have collected over 3000 ts mutants, probably including mutations in most or all cell-essential pathways, including about 200 new candidate cell cycle mutations. Subsequent molecular and cellular characterization of these mutants should provide new insights in plant cell biology; a comprehensive mutant collection is an essential prerequisite to ensure coverage of a broad range of biological pathways. These methods are integrated with downstream genetics and bioinformatics procedures for efficient mapping and identification of the causative mutations that are beyond the scope of this manuscript.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54831/>

Introduction

Phenotypic characterization of systematic mutant collections of model organisms is a proven approach for dissecting cellular complexity. The haploid unicellular green algae *Chlamydomonas reinhardtii* has a plant-like gene set, but it diverged from land plants before multiple genome duplications in the land plant lineage². In principle, lack of gene duplication and a mainly haploid life cycle greatly facilitates loss-of-function genetic approaches. However, targeted disruption of genes of interest is nearly impossible due to the lack of efficient homologous genomic integration. A random insertional disruption library is under construction, combined with identification of the disrupted site, so far yielding an arrayed set of 1,935 mapped disruptions representing 1,562 genes³. However, this approach (expected in general to produce null mutations) is not applicable to essential genes. Temperature-sensitive (ts) mutations can be recovered in essential genes, and recent methods allow efficient identification of the mutated gene and the causative lesion. Phenotypic analysis at high temperature then provides immediate information about the function of the mutated gene. We reported on the isolation and characterization of ts lethal mutations in ~70 essential genes in *Chlamydomonas*, focusing especially on genes involved in cell cycle progression and control^{1,4}.

Ts lethal screens have been a mainstay of genetic analysis in microorganisms for decades^{5,6}. In principle, a desirable feature is to approach "saturation," meaning that all genes capable of mutating to ts lethality are identified by at least one mutant, allowing a complete analysis. However, in practice, several factors limit the approach to saturation. First, while almost all genes can be mutated to loss of activity at high temperature, the efficiency of recovery of such mutants varies over at least an order of magnitude^{7,8}. Therefore, a random screen begins to pick up recurrent hits in "frequent flyers" long before saturation is approached. Second, while ts mutations usually result in reduction of function, they may not be true nulls at a restrictive temperature (and conversely, are frequently not fully functional at a permissive temperature). This problem can be dealt with to some extent by comparing multiple alleles; if they all share a common phenotype, this is more likely to reflect the result of simple inactivation of the gene. Multiple alleles are also very helpful for definitive molecular identification of the causative lesion¹. However, the "frequent flyer" problem means that multiple alleles in rarely hit genes can be difficult to recover.

For these reasons, we have been developing an enhanced pipeline to isolate and phenotypically characterize ts mutants. We have collected over 3,000 ts mutants so far, including about 200 new candidate cell cycle mutations. Molecular and phenotypic analysis of this collection, which already likely includes mutations in most or all cell-essential pathways, should provide new insights and hypotheses in plant cell biology. Importantly, this pipeline can be applied to any microorganism that grows on agar to efficiently construct ts mutant collections.

A note on equipment: two robots are very important for efficiency in this procedure (a colony picker and a combination replica plater/cherry picker). Pickers typically have metal pins. Picked colonies are held in air on these pins for some period (seconds to minutes, depending on the model). *Chlamydomonas* dies in about 20 sec on a metal pin in air. This restricts model choice for the organism. Accuracy matters. Our colony picker is reasonably accurate; however, it is somewhat violent in its action and has some possibility for spray-based cross-contamination. It is not used at higher than the 384 density (4.5 mm center-center spacing); at this density, the accuracy is completely acceptable. The replica plating/cherry picking robot (different attachments used for these applications) is much slower at picking, but is accurate enough for the 1,536 density (6,144 is a challenge, even for this very accurate robot; we have evaluated this density but elected not to use it because of its various difficulties). The robot will not work well if plates are loaded unevenly, etc. It is important to spot-check to be sure the right things are happening; of course, the robot will run unattended and in general, all will go well if the first few plates were correct.

Protocol

1. UV Mutagenesis

1. Prepare a batch of 100 - 200 rectangular agar plates with Tris-Acetate-Phosphate (TAP) medium^{9,10}. Prepare these plates a few days ahead and keep them on the bench to dry to ensure rapid absorption of the suspended cells in the next steps.
2. Culture *Chlamydomonas* cells up to 0.2 - 0.5 optical density (OD₇₅₀; ~ 2 days) in 100 ml of liquid TAP under light, at 25 °C and shaking at 100 rpm.
NOTE: UV mutagenesis is performed independently in two genetic backgrounds: Mat- Hygro^r (confers resistance to Hygromycin B) and Mat+ Paro^r (confers resistance to Paromomycin). Antibiotic choice is arbitrary, provided the two mating types have complementary drug resistances.
3. Check a sample of each of the cultures under the microscope to ensure that the cells are viable, healthy (swimming and intact), and without contamination.
NOTE: Overgrown cultures "crash" and lose viability, appearing as "ghosts" in phase contrast microscopy. Do not keep shaker cultures going once they reach saturation. The "ghost" phenomenon is easily detectable in step 1.3.
4. Dilute the culture to 0.003 OD₇₅₀. Wrap the bottle with aluminum foil to ensure homogenous density, as the strain is motile and swims directionally in response to light.
 1. Adjust the density of the suspension based on the planned UV dose, so that accounting for cell killing, 200 - 600 survivor colonies will form on the plates (**Figure 1**). See **Table 1** for information on UV exposure times.
5. Attach a small-tube cassette that fits a liquid dispenser and perform a series of washes for sterilization, according to the manufacturer's instructions, to prevent contamination.
6. Using an 8 x 12 liquid dispenser, dispense 4 x 96 drops of 2 µl each onto rectangular plates (**Figure 1**). Tap mildly at the edge of the plate to ensure the merging of all drops into a thin sheet of liquid and immediately cover the plates to prevent exposure to light.
 1. To ensure very even single cell dispersal, use dry plates, as mentioned above, and quickly cover the plates to prevent the cells from swimming in response to light. Keep the covered plates level and in the dark until all liquid is absorbed.
7. Place the plates under a germicidal UV lamp (30 watt germicidal UV tube) for periods of time determined empirically to give an optimal yield of ts- mutants among the survivors. Here, use times of 0.5 - 1.5 min at a distance of 40 - 50 cm to result in 90 - 99.9% killing. Survivors contain 100 - 1,000 UV-induced point mutations, of which ~ 10% change coding sequences.
 1. In order to ensure maximum potency of UV irradiation with no reversion due to light-dependent DNA repair^{11,12}, work in the dark at this step and immediately pack the plates in a dark box after irradiation.
8. Keep the plates in the dark for 8 - 24 hr at room temperature.
9. Place the plates in a 21 °C incubator with illumination to form colonies. Colony formation takes ~10 days. Make sure to wrap the plates with plastic bags and add absorbing paper inside to blot up liquid condensation. Evaporation and condensation cycles otherwise can provide liquid film routes for contaminants to enter the plates.
10. Load the plates in the relevant stacks as sources for robotic colony picking (**Figure 2**). Pick colonies for 384-arrays on rectangular plates, and grow them at 21 °C with illumination (~ 1 week).
11. Condense the 384-arrays into a 1,536-arrays (4:1) using a replica-plating robot (**Figure 3**), and allow the plates to grow for ~ 3 days in the 21 °C incubator.
12. Replicate the 1,536-arrays to two plates each and place one in the 21 °C incubator (permissive temperature) and the other in a 33 °C incubator (restrictive temperature). Following 24 hr, replicate the plates in 33 °C to a new set of pre-warmed plates, and place them in the 33 °C incubator.
NOTE: The reason for this secondary plating is that ts lethal mutants can in some cases accumulate substantial biomass, even if the cells are arrested in the first cell cycle after plating. The secondary replica eliminates this signal and greatly increases sensitivity.
13. Photograph the plates with a digital camera following 3 days of growth at 33 °C and 5 days of growth at 21 °C. Hold the plates in a fixed frame. Use a "grid-plate" marked with nine alignment indicators that is photographed together with the culture plates (**Figure 4**). Photograph culture plates as alternating paired 21 °C and 33 °C (21/33) images.
NOTE: Different incubation times are used to equalize growth since the wild type grows significantly faster at 33 °C than at 21 °C.

2. Identification of ts Mutant Candidates: First Screen

1. Process the paired 21/33 plate images with a custom Matlab image analysis software to eliminate the background and to segment the images into a 1,536-array. The program will determine the detected biomass (total pixel intensity) in each position (**Figure 5**).
NOTE: The software (provided in the S.I. along with instructions) will use the grid-plate image to determine the locations of cells (individual entries) in a 1,536-array at the magnification and plate alignment used and calculate total pixel intensity in each cell. These values for each mutant are then compared against adjustable parameters to determine required growth at 21 °C relative to a ts+ standard and the degree

of temperature-sensitivity, defined as: $S(\text{Mut}_{33})/S(\text{WT}_{33})/S(\text{Mut}_{21})/S(\text{WT}_{21})$, where S is signal (pixel intensity after background subtraction), Mut is an individual mutant, and "WT" is a randomly chosen non-temperature-sensitive colony (mutagenized strain that had ts+ phenotype). Growth at 21 °C is defined as $S(\text{Mut}_{21})/S(\text{WT}_{21})$. In this first screen, apply relaxed selection criteria (allowing relatively low growth at 21 °C and a relatively low degree of temperature sensitivity) to keep the false negative rate low.

2. Load the list of selected colonies generated by the software as an instruction file for the single-colony picking robotics (typically into a 384-array). Prepare the source and target plates according to the robotics instructions and pick colonies to array (**Figure 6**).
NOTE: Conventional pickers require an instruction file in some format, such as .csv, .txt, or .xls. All pickers will have the capacity to be file-driven; different formats will require minor editing of the MATLAB code (source code provided).
3. Place the target plates in the 21 °C incubator for ~ 5 days to grow a stock plate.

3. Identifying ts Mutants: Second Screen

1. Repeat step 2.1 to retest picked colonies. Typically 30 - 50% of colonies will retest as clear ts lethals, for a yield of 2% - 5% ts lethals relative to initial colonies surviving UV mutagenesis.
2. Repeat step 2.2 for picking the twice-screened ts lethals, but with a modified instruction file designed to array colonies into blocks of 100 colonies on rectangular plates at a 384-density. This is for convenient microscopic examination in the next step. The instruction file format is specified at runtime by the MATLAB code.
3. Make sure to include several WT colonies as a control and place the plates at 21 °C for ~ 5 days.

4. Initial Phenotype Determination

1. Replicate the 100-block plates arrayed in step 3.2 and place them in the 21 °C incubator for ~ 2 days to obtain freshly growing colonies.
2. Replicate the fresh copy of the 100-block plates (4.1) to three copies. Place one copy in the 21 °C incubator and one in the 33 °C incubator as controls.
3. Place the third copy ("screening plates") in a robotic setup, and spot the colonies with long pins touched with sterilized water.
NOTE: Chlamydomonas cells pinned to agar robotically tend to land initially as a rather dense spot of cells, with few single cells available for microscopic inspection. To optimize the colonies for microscopic inspection, spot the initially transferred cells with a drop of water (the volume of water transferred by the robotic long pins is ~ 100 nl). This results in dispersion of the cells in a small radius (~ 1 mm) about the initial pinning center, with many isolated single cells.
4. Take photomicrographs of a region of each spot of the screening plates at time 0 (while still single, undivided cells) (**Figure 7**) and place the plates at 33 °C for incubation. Determine image location by the manual stage control of a modified tetrad dissection microscope set to give stops at the 4.5-mm center-to-center spacing of a 384-density array.
5. Remove the screening plates from the 33 °C incubator and take photomicrographs, as in step 4.4, at varying time points (10 hr, 20 hr, 48 hr). Make sure the stage, the plate holder, and the stage controller are precisely calibrated to get images of the same cells in every time point. Perform quick photomicrograph acquisition (~ 10 min).
6. Use the second copy in the 33 °C incubator to verify the ts phenotype and to make sure that temperature fluctuations during image acquisition have no major effect on ts phenotype.
 1. Analyze microscopic images and select for mutants based on the desired criteria (**Figure 8**). Spot the final selected set in a 96-arrayed agar plate. Make sure that each plate contains mutants of the same mating type and drug resistance.
 2. For each plate, incorporate the last two columns a positive (query mutation) and a negative (WT) control for the complementation assay.

5. Complementation and Linkage Testing of New Mutants to "Frequent Flyers"

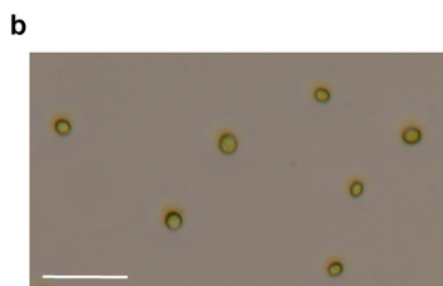
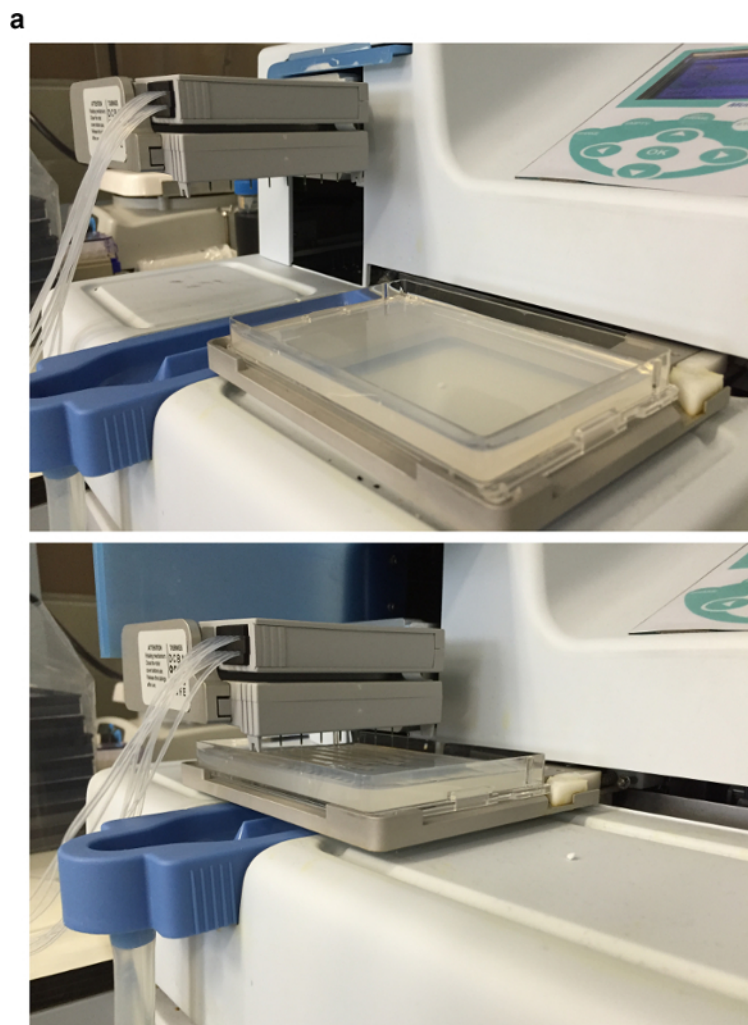
1. Transfer large amounts of the arrayed colonies to nitrogen-free gamete-induction medium¹⁰ in 96-well microplates (density of each colony should be around 0.2 O.D.). Incubate the plates under light (13 - 20 W mercury bulbs) for ~ 5 hr to allow gametogenesis.
NOTE: This step can be conducted either with a replicating robotic setup or manually with a simple plating device.
2. Suspend queries with the opposite mating types harboring the alternative resistance cassettes into tubes with nitrogen-free gamete-induction medium¹⁰ for gametogenesis.
3. Mix the samples in a target plate in a mating mixture volume of 20 µl (**Figure 9**). Following ~10 min under the light, spot 5 µl from each well twice: once on a TAP plate for linkage testing and once on TAP + 5 µM Paro + 9 µM Hygro for complementation testing.
4. Incubate the linkage plates in the 21 °C incubator overnight, and then wrap them in foil. Keep them in the dark for 5 days to allow zygospore formation.
5. Incubate complementation-testing plates for ~10 days in the light at 21 °C.
NOTE: The drug amounts are calibrated to allow survival of doubly heterozygous diploids, but they are still enough to eliminate the mating haploids. These amounts were calibrated for standard resistance cassette integrations used in this specific project; with new integrations, doses should be recalibrated. Most biomass is verified to be diploids by flow cytometry (**Figure 9**), although a variable level of haploids (probably from meiosis and growth of doubly resistant segregants) is usually observed as well.
6. Replicate the complementation-testing plates into two copies for ts- phenotype identification. Place one copy at 21 °C and one copy at 33 °C for ~ 5 days.
7. Test colonies for the ts- phenotype, as described in section 2.1 (**Figure 5**). Colonies that are not complementing with the query likely represent alleles of the same gene as the query (diploids hetero-allelic for mutations in the same gene).
8. For linkage testing, following step 5.4, shift the plates back to light to allow meiosis and outgrowth of haploid segregants for ~7 days.
9. Replicate linkage testing plates to TAP + 10 µM Paro and 10 µM Hygro to select for double-resistant progenies (predicted to be 25% of the haploid progeny, since the cassettes are unlinked) and incubate at 21 °C for a week.
NOTE: Increase the drug dosage for doubly resistant haploids.
10. Test colonies for the ts- phenotype, as in step 2.1.

NOTE: A ts- phenotype is expected (and observed) for mutants in the same complementation group as the query. In addition, a ts- phenotype in the linkage assay, for a mutant that complements the query, can reflect tight linkage between the query and the new mutation. Mutants that complement the tested queries are likely new genes that will enter the pipeline for bioinformatic identification of the causative lesion, and ultimately for further phenotypic analysis.

Representative Results

We show an accelerated pipeline for isolating ts mutants in *Chlamydomonas*. Cells are dispensed on agar plates, and shortly after quick validation under the microscope for single-cell density, plates are UV irradiated (**Figure 1**). Typical irradiated colonies are identified and picked into an arrayed format after 10 days of growth at a permissive temperature (**Figure 2**). The resultant plates in the 384 format are merged to a 1,536-array (**Figure 3**). From these first steps of collecting irradiated colonies, we have arrayed ~ 200,000 colonies so far. The density of the suspension is adjusted based on the planned UV dose so that, accounting for death, 200 - 600 survivor colonies will form on the plates. Three UV exposure times (1.5 min, 1 min, and 0.5 min) and three densities were tested accordingly (**Table 1**). Empirically, the 1.5 min exposure time yielded most of the ts- mutants (~ 50%); however, by far, 1 min yielded most of the cell cycle candidates. Therefore, future UV mutagenesis rounds were done with 1 min only. An important concern is the splashing during initial picking and the cross contamination (especially in the high-density formats). This may result in duplication and further phenotyping of the same mutant twice. To minimize the probability for such a scenario, take care to collect colonies at the optimal size, and make sure that adjacent colonies are not picked in the initial assay.

Next, two sequential ts- phenotype assays (**Figure 5**) were performed, and around 3,000 ts- mutants were isolated and phenotypically characterized by time-lapse microscopy (**Figure 8**). Due to the biological focus in studying the cell cycle of interest in phenotypes that meet certain criteria, we are not interested in collecting more than two alleles in each target gene. Therefore, we performed complementation and linkage testing with already characterized genes with more than two alleles (Query) against newly collected candidates to remove highly recurrent genes from the downstream pipeline (**Figure 9**).




 Validation of single cell plating by microscopy followed by UV irradiation.

Figure 1: Uniform Spreading of Unmutagenized Cells and UV Mutagenesis. (a) 384 x 2 μ l drops are dispensed on an agar plate using a reagent dispenser. (b) Random plates are tested under the microscope to verify single-cell density and are UV irradiated with a 1-min exposure. Scale bar = 50 μ m. [Please click here to view a larger version of this figure.](#)

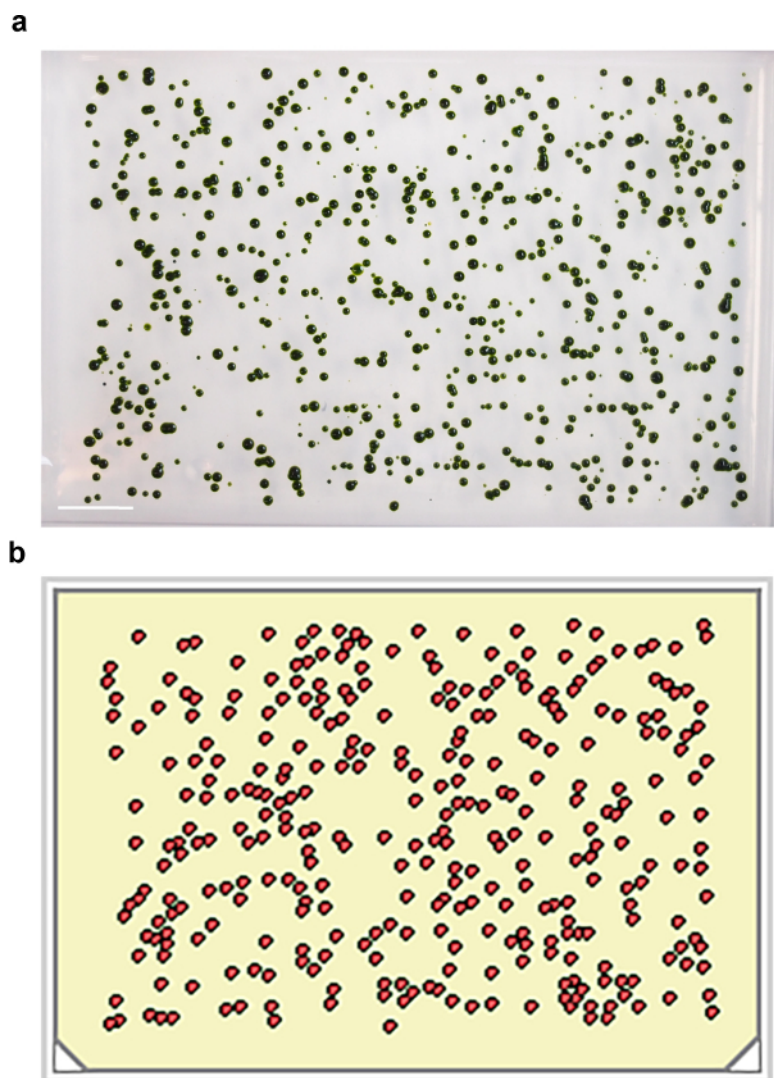


Figure 2. UV-mutagenized Colonies Picked for the 384-arrays. (a) UV-irradiated single cells are grown for ~10 days into visible colonies. Scale bar = 2 cm. (b) Image analysis program recognizes separable colonies according to certain parameters and robotically arrays them into 384 plates. [Please click here to view a larger version of this figure.](#)

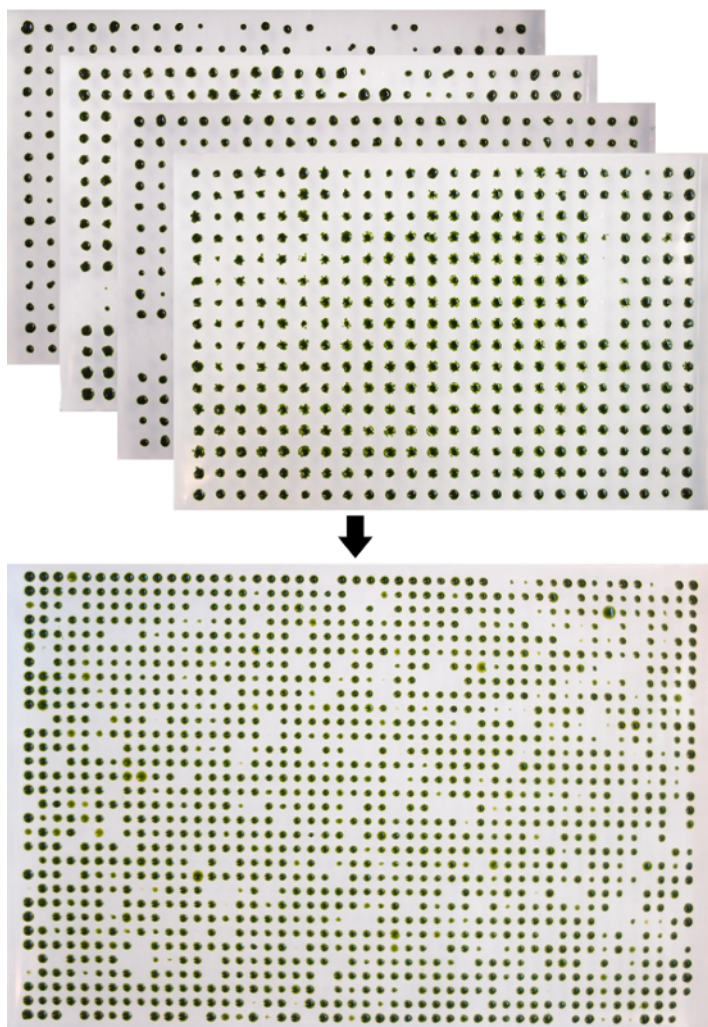


Figure 3. Merging to the 1,536-array. Four 384-format plates are merged to one 1,536-plate; once grown, they are replicated again to test for the ts- phenotype. [Please click here to view a larger version of this figure.](#)

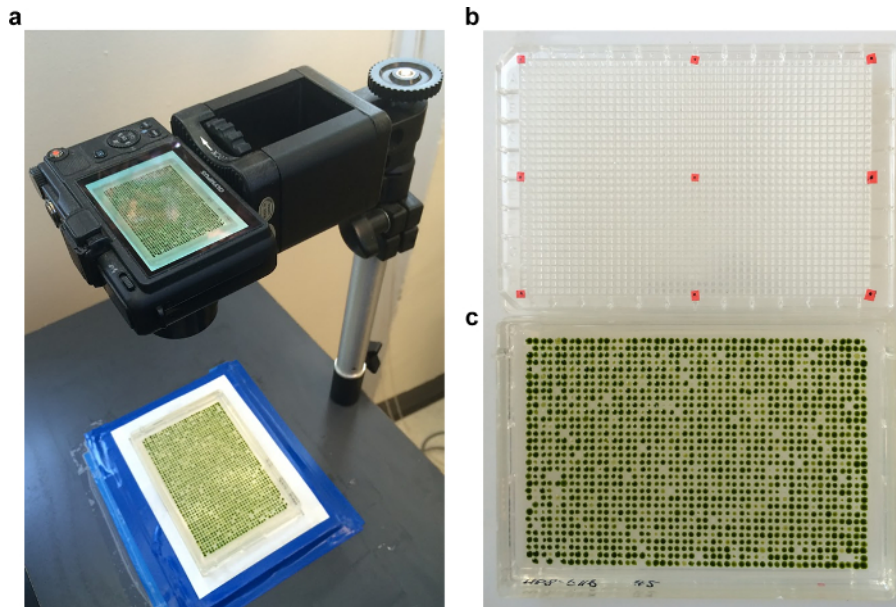


Figure 4. Imaging for Quantification. (a) Plates are held in a frame under a document photography stand so that all plates in a series are photographed at identical magnification and positioning. (b) The first image is of a 1536-well microtiter plate with red dots placed at corners and midpoints. This "grid-plate" image defines the position of the array. (c) Example of a 1,536-array after incubation. [Please click here to view a larger version of this figure.](#)

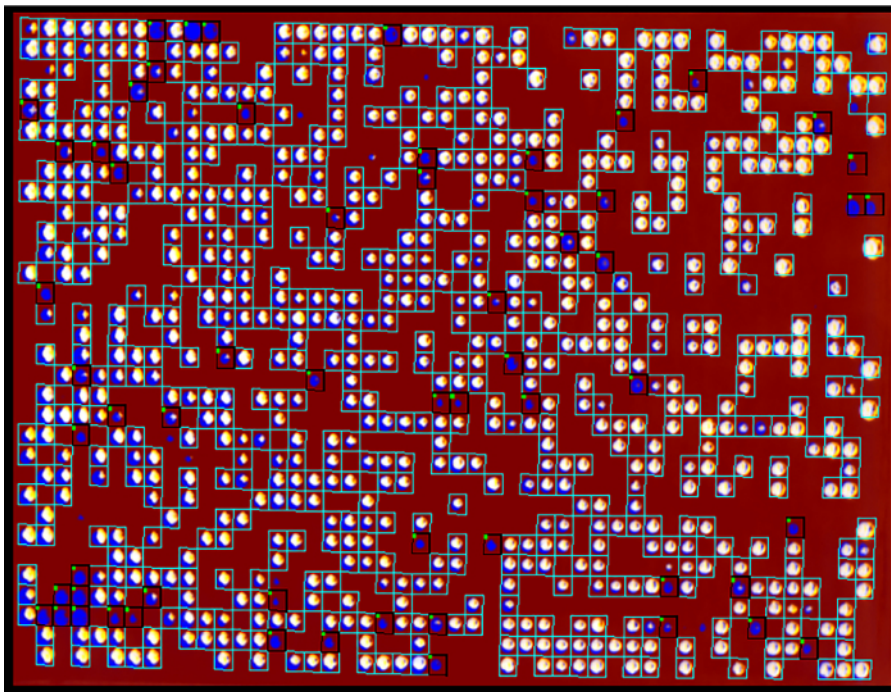


Figure 5. Identification of the ts- phenotype by Semi-automated Image Analysis. Plate images are analyzed by custom MATLAB software (provided in the S.I.). The image is automatically segmented into cell arrays (96, 384, or 1,536), and the signal in each cell is quantified. Growth at 21 °C is marked in blue, and growth at 33 °C is marked in yellow. Colonies exhibiting significantly higher growth at 21 °C compared to 33 °C (standardized to ts+) are selected and marked in black squares with a green dot. These choices can be edited manually. Final selections are transferred to a file used by the colony-picking robot. [Please click here to view a larger version of this figure.](#)

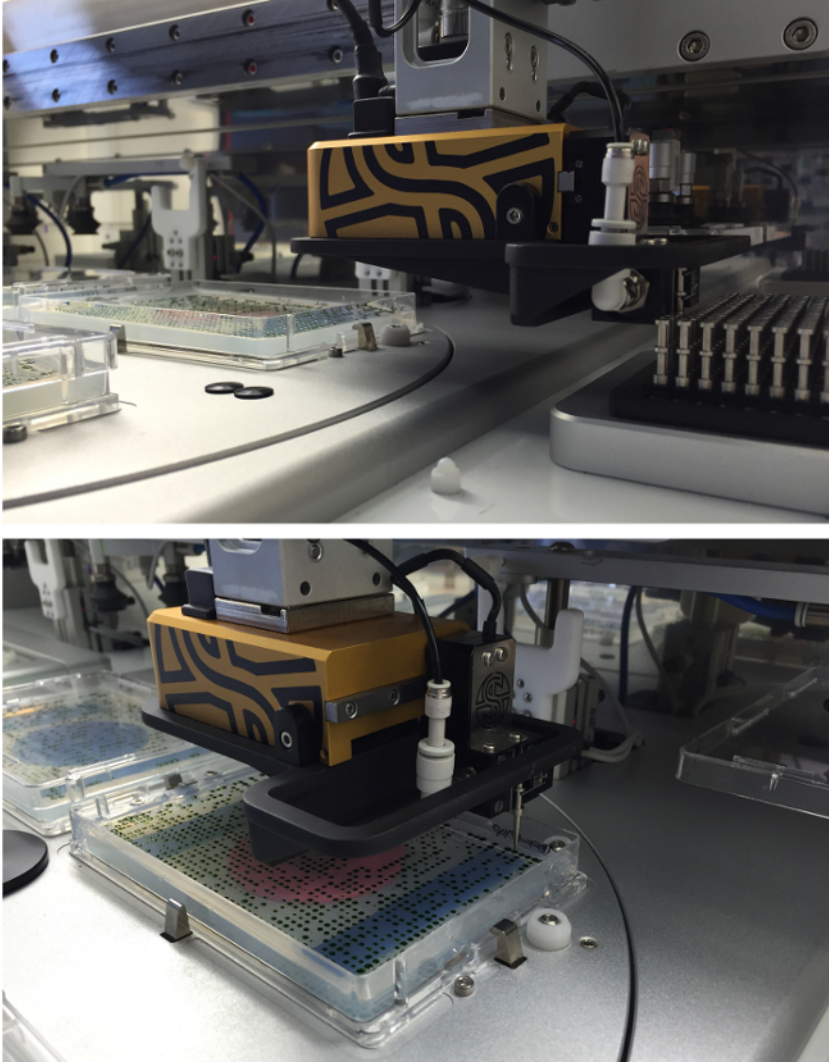


Figure 6. Robotic Picking of First-round *ts* Lethal Candidates. The cherry-picking robot follows instructions from a file generated as described in step 2.1 to pick candidates for a new array. The top panel shows the loading of a sterilized pin. The bottom panel shows the precise picking from a certain colony in the source plate. [Please click here to view a larger version of this figure.](#)

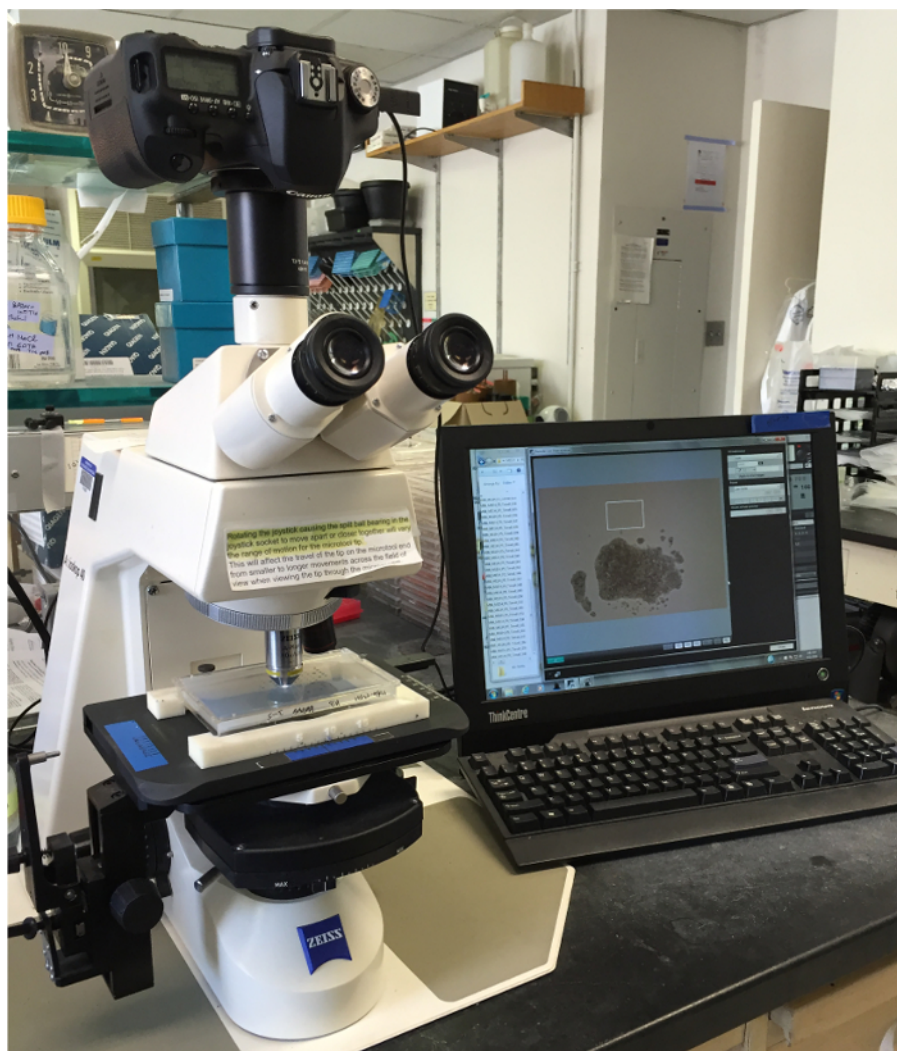


Figure 7. Time-lapse Screening for Initial Sorting of *ts* Lethal Phenotypes. Clones that passed two rounds of the screening protocol illustrated in **Figure 5** are replicated to plates at a cell density such that individual cells can be resolved microscopically. A modified tetrad dissection microscope is used to identify positions at which photomicrographs are taken after 0 hr, 10 hr, and 20 hr incubations at 33 °C. [Please click here to view a larger version of this figure.](#)

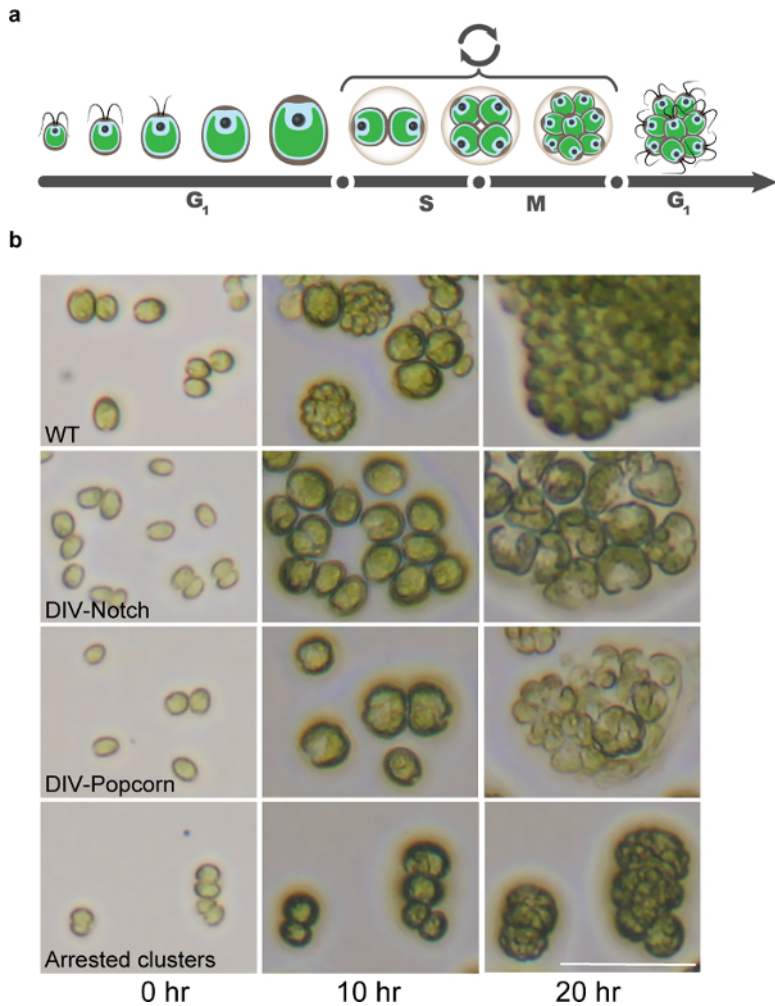


Figure 8: The Chlamydomonas Cell Cycle Mutants Identified by Time-Lapse Microscopy. (a) Illustration of the Chlamydomonas unique cell cycle describes the long G₁ phase characterized by cellular growth, followed by fission S-M cycles, which end up with the hatching of newborn daughter cells. (b) Microscopic images demonstrate WT cells during the cell cycle and the identification of typical cell cycle mutants that fail to complete mitosis. Scale bar = 50 μ m. [Please click here to view a larger version of this figure.](#)

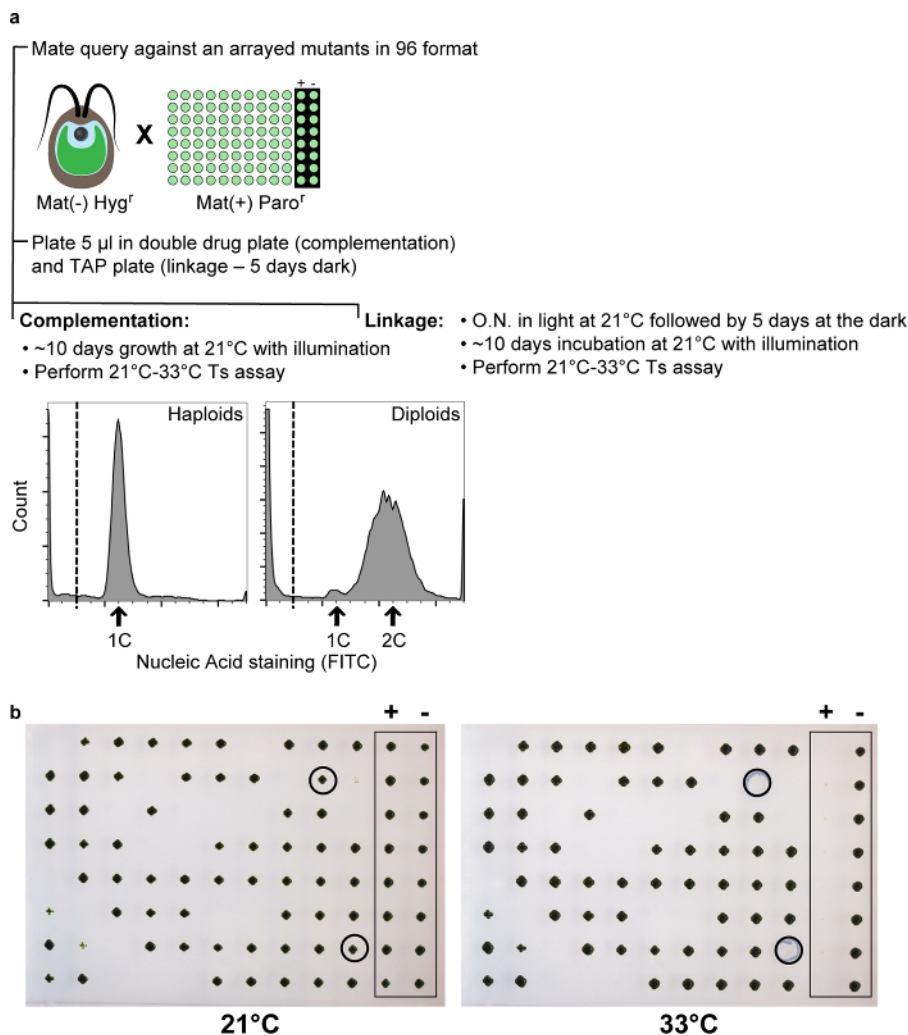


Figure 9: Complementation and Linkage Test. (a) An antibiotic resistant (e.g., Hygro) query with a frequently repeated mutated gene is crossed in a 96-well plate with a set of mutants of the opposite mating type that harbor a second antibiotic resistance cassette (e.g., Paro). Complementation plates yield high fraction of diploids as shown by the nucleic acid staining and analysis in flow cytometry (lower left panel in a). (b) The mating mixture is tested both for complementation in diploids and for linkage in double-resistant meiotic progeny. The positive control is the query itself in the opposite mating type, and, as expected, shows the ts- phenotype at 33 °C, whereas the negative control is WT and shows the ts+ phenotype. The circled colonies show no complementation with the query and are therefore new ts- alleles for the query gene. These are generally excluded from further characterization, since only "frequent flyers" are in the complementation test set. [Please click here to view a larger version of this figure.](#)

Time	O.D.	Colonies picked (#)	Ts- phenotype	Cell-cycle candidates
1.5'	0.012	6000 (Avg=~200)	200 (3.3%)	7 (3.5%)
1'	0.003	11,000	131 (1.19%)	15 (11.4%)
		(Avg=~360)		
0.5'	6E-04	9000	40 (0.45%)	1 (2.5%)
		(Avg=~300)		

Table 1: Calibration of Irradiation Times to Maximize the Yield of Cell Cycle Mutant Candidates. Three irradiation times were tested (30 plates each) with corresponding ODs to ensure surviving colony numbers (200 - 600).

Discussion

The pipeline described here for high yield isolation of ts lethal mutants ensures that presumably all cellular-essential pathways of the Chlamydomonas genome are represented. The two most critical steps for efficient collection of potential cell cycle genes and for the elimination of repetitive "frequent flyer" alleles are: 1) the coherent definition of arrest phenotype characteristics for incomplete cell cycles and 2) the parallel complementation assay against already-identified query genes to enlarge the collection with newly isolated ones.

When synchronized by light-dark cycles, *Chlamydomonas* grows photosynthetically during daylight hours and increases in cell size > 10x without any DNA replication or cell division¹³. Approximately coincident with the onset of night, cells then undergo multiple cycles of alternating DNA replication, mitosis, and cell division (**Figure 8**). This regulatory scheme provides a natural distinction between genes primarily required for cell growth and integrity and genes required specifically for the cell division cycle. We found that the 10-hr and 20-hr time points are very informative for an initial rough phenotypic cut¹. The broad classes of ts lethal mutants that we recognize currently, based on these images (see S.I. in Tulin and Cross, 2014)¹, are: Notch, Popcorn, Round, Small, Medium, early lysis, and multiple-cycle (**Figure 8**).

The three most relevant categories we focus on are Notch, Popcorn, and Round. The "Notch" and "Popcorn" phenotypes were shown previously to be characteristic of most cell cycle-specific lesions (e.g., mitotic cyclin-dependent kinase, DNA replication machinery, and Topoisomerase II)¹. The appearance of one (Notch) or multiple (Popcorn) apparent planes of incipient but unsuccessful cell division is a convenient morphological indicator of cell cycle initiation. These mutants generally exhibit little or no growth defects, with increases in cell volume similar to WT at the 10-hr mark. The Notch and Popcorn phenotypes are evident at 10 hr and are fully developed (frequently associated with cell lysis) by 20 hr. "Round" cells grow similarly to WT but with much-reduced production of apparent incipient division planes, thus yielding large, round arrested cells. Previous mutants in this category have fallen into components of the anaphase-promoting complex¹⁴ or in genes required for microtubule function (tubulin-folding cofactors, gamma-tubulin ring complex)¹. At later times, these cells frequently exhibit pronounced cell lysis.

"Small" and "Medium" cells grow either negligibly (Small) or significantly less than WT (Medium). Many of these mutants identified to date have lesions in genes whose annotations suggest roles in basic cellular growth processes (translation or membrane biogenesis). The main microscopic discrimination between Medium and Round rests on the amount of growth at 10 hr (Round: like WT; Medium: reduced). Because the Small and Medium categories are quite large and probably reflect lesions in a great range of cellular pathways, we are not attempting to saturate these categories; however, we do want to molecularly identify representatives of the class to understand phenotypes of loss in diverse pathways. Two unstudied categories are: 1) the early-lysing mutants that lose integrity (loss of green color, loss of refractility) by the 10-hr mark, with little evidence of prior cell growth and 2) the multiple cycles. Cells proliferate similarly to WT at 10 and 20 hr, though they exhibit a complete inability to carry out longer-term proliferation.

We are mostly characterizing "notch," "popcorn," and "round" and exclude small and medium round cells, as well as leaky mutants that complete few cell divisions. This is principally to ensure that basic cellular features, such as growth and membrane integrity, are functional and enrich the probability for division-related genes. This approach is found to be empirically efficient; however, it may be that a cell cycle gene is pleiotropic and has additional roles earlier in G1, before actual division. Such cases, which we expect to be rare, are missed. More generally, we aim for homogenous arrest, which in high probability is due to one causative mutation that is a completely dysfunctional protein. However, for the same reason as just described, there may be several arrest points and therefore, some flexibility is advisable in choosing candidates.

In order to enrich the collection with newly identified genes, the chosen candidates are assayed for complementation. We require ts- in the positive control (query against query mutation) and ts+ in the negative control (query against WT). New mutants in the same complementation group as the query are ts-. Membership in the same complementation group almost invariably reflects a molecular lesion in the same gene (this has been the case for every such gene we have tested). Therefore, for "frequent flyers," this criterion is exclusionary for further characterization. Mutants that were not at the same complementation groups as the tested queries are candidates for new genes and are further characterized by bioinformatics and experimental tools. Highly variable recovery of ts- alleles into different complementation groups is a well-known phenomenon—that is, variability is markedly greater than Poisson noise, due to the great intrinsic variability of mutability to ts- between different genes. Causes could include intrinsic thermolability differences; different protein sizes; the presence of a protein as a monomer versus as a large, stabilized complex; and mutagenic hot spots. This is almost a pure nuisance. However, one resulting favorable outcome is that the "frequent flyer" list is not long (with only a few targets occupying most of the list), so the labor-intensive complementation testing is not a massive undertaking until the later stages of the project.

As a complementary approach, we performed a linkage assay. In this assay, double-resistant progenies are selected and are tested for the ts- phenotype. A ts- phenotype is expected (and observed) for mutants in the same complementation group as the query or for tightly linked mutations. For each of the tested genes, a WT progeny is expected to appear (ts+ phenotype) in a certain probability depending on the genetic distance. We estimate that there are around 100 zygospores for each mating in these spots. Assuming 100% meiotic efficiency, this will result in around 100 double-drug-resistant progeny from the unlinked drug resistance cassettes (25% of the meiotic progeny, four per meiosis, due to Mendelian inheritance). This would also be the case for ts- mutations, where 25% of the progenies will be double-mutant, and 25% will be WT if the query and test mutations are unlinked. Therefore, out of the double-drug-resistant progeny, 25% will be WT (around 25 cells). This is the case for completely unlinked mutations; however, moderate linkage (within ~ 20 cM, about 2 Mb, or 2% of the genome¹⁵) will strongly reduce or eliminate the ts+ signal. In the case of linkage of the tested mutation to the antibiotic cassette, ts+ haploids that are double-drug-resistant are present in very low amounts. This manifests as apparent failure to recombine with all mutants tested, despite complementing all mutants tested, an aberrant result that is easily noted; in such cases, backcrossing will solve the problem.

Both from prior knowledge and from sequence analysis, we expect cell cycle genes in the *Chlamydomonas* to be around 500 genes², although most, but probably not all, are essential. We will evaluate the necessity for additional mutagenesis rounds as more mutants are collected and the level of saturation rises.

This procedure is uniquely designed for studying essential biological processes and the genes and proteins that carry them out. Other methodologies to generate perturbations in essential genes exist (e.g., transformation of randomly mutagenized alleles¹⁶, conditionally transcribed alleles¹⁷, or hypomorphic alleles¹⁸). However, they all require homologous recombination, which is strongly suppressed in vegetative *Chlamydomonas*. The clustered, regularly interspaced short palindromic repeat (CRISPR)/Cas9 system has been established as a powerful tool for gene modification¹⁹; however, it is yet to work efficiently in *Chlamydomonas*²⁰. Critically, all of these methods require prior knowledge of the target. This is a severe restriction if one wishes to have the possibility to learn something new! Our approach will yield mutations identifying essential genes, independent of any prior knowledge. Therefore, at the present level of technology, isolation of random ts mutations followed by gene identification by deep sequencing may be the most efficient method of gaining rapid entry into microbial cell biology in the plant superkingdom.

Identification of causative mutations (from among ~100 coding-sequence-changing mutations in each clone) is beyond the scope of this paper. Deep sequencing of bulked segregant pools¹ is effective but labor-intensive. A combinatorial pool strategy for the determination of all mutations in a large number of strains, after sequencing a small number of pools, is very cost- and labor-effective. A new strategy for combinatorial bulked segregant sequencing is under development that will allow the identification of causative mutations in dozens of mutants simultaneously in a single sequencing run (in preparation). These efficiencies are very important to allow the critical gene identification step to keep pace with the very rapid accumulation of mutants that is made possible by the procedures described here.

Disclosures

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