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Sample Preparation for Single Particle Cryo-EM

Part 1: Preparing your sample for vitrification

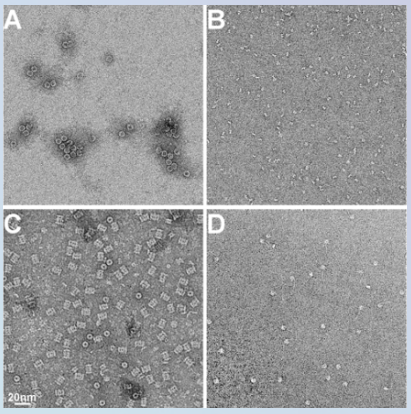
Sample preservation in vitrified ice (vitrification) is widely acknowledged as the first step in the cryogenic electron microscopy (Cryo-EM) workflow. However, prior to sample preparation researchers must consider the sample quality and determine its suitability for high resolution structure determination. A good understanding of these key considerations along with the right instrumentation is vital to beginning any CryoEM project and its subsequent success.

Protein Purification

Before pursuing a cryo-EM structure it must be determined that the sample purity is high (>99%, a single band in SDS-PAGE gel), monodisperse in solution and homogeneous in nature (single peak in SEC profile). Particle heterogeneity, both compositional and conformational can lead to difficulty in producing particle image "classes" necessary for building 3D reconstructions from the 2D data. This loss of information limits the quality of the resulting 3D maps and therefore the answerable biological questions.

The biological specimen of interest should remain active in a suitable buffer with optimized conditions. When necessary or after storage, functional assays to determine protein activity and thermostability can be utilized. Vitrification requires low volumes of several microliters and depending on the sample, concentrations from 50nM to 5µM.

With advancements in software and data analysis the single particle workflow can deal with partial heterogeneity and some sample impurity. However, obtaining a solution of isolated homogeneous particles of interest is the best guarantee of a successful high-resolution structure.



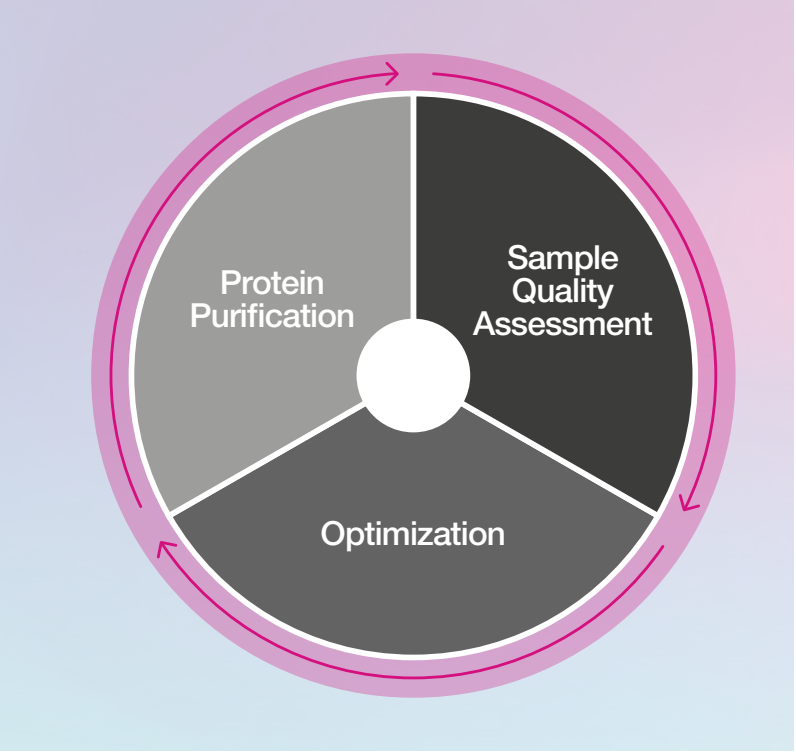
Examples of EM Images of negatively stained protein samples. A) Horse spleen ferritin, B) fragment of antigen binding (Fab), C) archeal 20S proteasome and D) nucleosome. All images at same magnification. The scale bar is 20nm

Assessment of Sample Quality

It is good practice to assess sample quality and homogeneity prior to vitrification. In addition to biophysical methods like SDS-PAGE, SEC and dynamic light scattering (DLS), the determination of sample homogeneity is routinely accomplished visually using negative stain transmission electron microscopy (nsTEM). This allows the qualitative examination of the particle concentration, composition, and conformational state by fixing the sample solution with stain (e.g., uranyl formate) to a carbon support film on an EM grid. These negatively stained specimens can then be loaded one at a time and examined in simple to use side-entry holder microscopes widely available in EM core laboratories. However, training is required to identify particles of interest and the conditions for "good" and "bad" staining. The time required to perform this analysis is minimal and it provides insight into the specimen quality on the microscopic scale.

Instrumentation

The instruments and supplies needed for these methods are readily available in most biochemistry labs and EM core facilities. It is always recommended to optimize sample quality to as high a degree as possible prior to vitrification. Having access to EM specimen prep and analysis nearby to biochemical workflows makes this faster and easier to achieve.



Once biochemical readiness has been determined the sample optimization for plunge freezing can begin. In the next post we will look at the considerations and methods for optimizing samples for conventional sample vitrification for cryo-EM.

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K.A. Taylor, R.M. Glaeser Retrospective on the early development of cryoelectron microscopy of macromolecules and a prospective on opportunities for the future doi: 10.1016/j.jsb.2008.06.004 Published: June 18, 2008

Part 2: Conventional Plunge Vitrification Optimization

The preparation of acceptable frozen-hydrated samples on transmission electron microscopy (TEM) grids is well known to be a challenging bottleneck in the cryogenic electron microscopy (cryo-EM) workflow. During sample vitrification it is assumed that the solution contains biological macromolecules that are distributed randomly and in suspension. Until recently it was generally accepted that the sample particles remained identical to those in bulk solution. However, in most samples prepared using conventional techniques we know that this not the case. Purified and biochemically optimized sample may not survive the standard method of blotting to make thin aqueous films for vitrification. The result is a necessary trial and error workflow using a series of experimental adaptations to find the optimal conditions for each unique biological specimen.

Conventional Methods

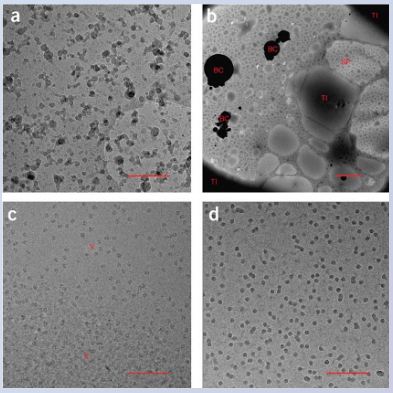
The conventional method of specimen preparation starts with the application of sample to a TEM grid followed by a blotting step to remove excess liquid, forming a thin aqueous film suitable for plunge vitrification into liquid ethane. The idealized specimen assumes the resulting distributions of particles in the thin vitreous layer are identical to that of bulk solution in areas where the ice itself is neither too thick nor too thin. In practice, initial success is rare, and optimization is often necessary to deal with ice variability and detrimental sample dependent behavior.

The standard cryo-EM workflow involves an iterative process of freezing many grids of varying quality and then screening for successful specimen using a cryogenic electron microscope. Since microscope resources can be expensive and hard to come by, the resulting costs and prolonged research cycle limits the efficiency of specimen optimization pathways.

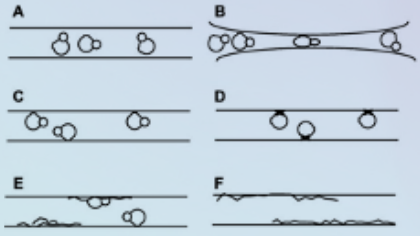
Reasons for Failure

Plunge freezing by conventional methods requires the manual handling of small fragile grids under liquid nitrogen conditions. The quality of resulting specimen grids is often user dependent leading to poor consistency. Furthermore, experience with cryo-EM is necessary to determine ‘good’ or ‘bad’ sample quality as outcomes are inconsistent and varied in nature.

It is a well-known good practice to avoid air bubbles during the handling of bulk protein solutions due to denaturation at the air-water interface (AWI). In the context of thin film formation for sample vitrification, the increased AWI surface area leads to 1000 or more AWI collisions per second for specimen thickness at 100nm or less. Therefore, it is likely that particle interaction with the AWI is the main cause of issues resulting from extremely thin sample formation on the specimen grids. Those undesirable issues being limited particle orientation and distribution, degradation, and aggregation.



Sample cryo-EM images exhibiting poor quality due to (A) ethane contamination, (B) Non uniform ‘splotchy’ ice and ice contamination, (C) non-vitreous crystalline ice, and (D) freeze dried particles.



Representations of the differences between desirable preparation outcomes relative to the AWI, (A), and a variety of undesirable outcomes (B-F)

Due to advancements in microscope imaging and data analysis software we have more information available to assess the underlying causes of sample preparation failure. Air water interface interactions have been experimentally determined to be ubiquitous to samples prepared using standard methods. Conventional sample preparation for cryo-EM requires optimization in almost all cases and none of the existing optimization methods works consistently well for different kinds of specimen.

Optimization Pathways

There are a variety of experimental methods meant to deal with standard specimen preparation failures for the sample at hand. However, there are not yet any optimization workflows specific to sample type. Only a consensus that optimization is required for most samples and that approximately 10 different methods will be attempted in combination before improvement is seen. Additionally, none of the currently available methods can be determined to work in advance of sample preparation. Although in experts' hands, experience can lead to a few common-sense first steps.

The Future of Sample Preparation for Cryo-EM

Next-generation sample preparation available on chameleon employs a blotless method, automation, and speed to outrun the effects of particle adsorption to the air water interface while standardizing ice quality and thinness in the hands of novice users. Optimizable vitrification for unique sample behavior can reduce the time and costs associated with trial-and-error experimental optimization necessary using traditional methods.

In the next post we will explore how characterization of the unique sample dependent effects of the air-water interface can lead to efficient optimization pathways and open doors to sample specific workflows for cryo-EM specimen prep.

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Part 3: Next Generation Sample Preparation

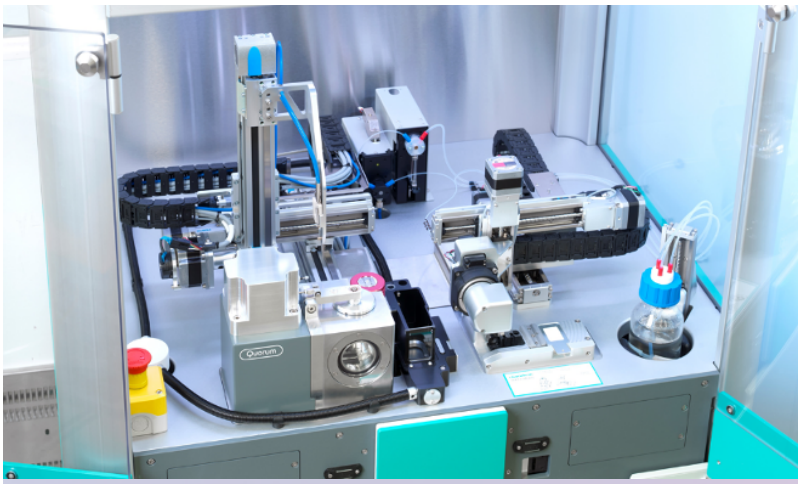
The period responsible for the current generation of cryogenic electron microscopy (cryo-EM) instrumentation and image processing software is often referred to as the "resolution revolution." The results of these technological advancements have been achievements in higher quality structures and cryo-EM becoming the go-to method for structural biologists. While it has been demonstrated that conventional plunge-freezing can achieve atomic resolution on the latest electron microscopes with ideal specimen, real world results still struggle to break the resolution barriers needed to answer the detailed biological questions at hand. To keep pace with the rapid advances in current hardware and software capabilities, next generation sample prep methods are realized on emerging instruments such as the chameleon to minimize endless sample optimization workflows and enable routine high-resolution reconstructions.

Variability Control

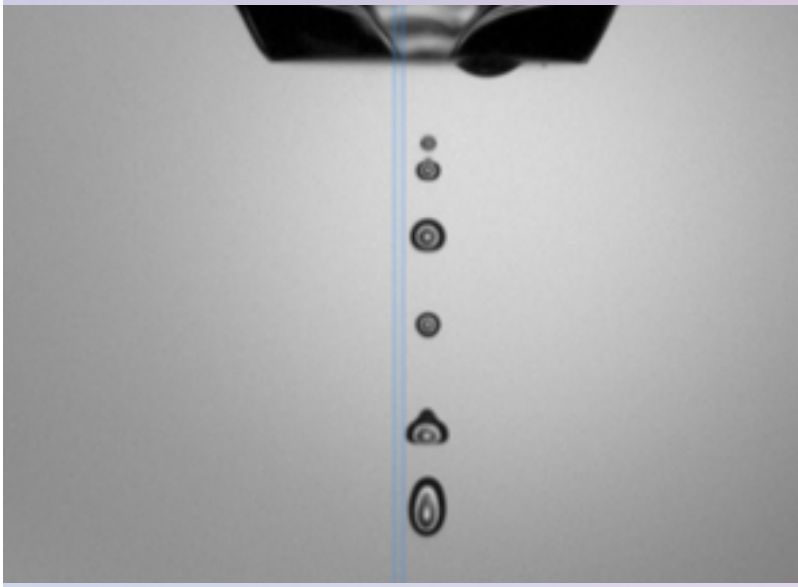
Bringing together, robotics, automation, and intelligent software allows experts and novice users alike quicker access to consistent results. Users can confidently work with the instrument through a terminal and control the process with guided software protocols intended to handle the experimental framework and limit manual intervention.

The chameleon achieves this automation through the novel design of picolitre dispensing onto self-wicking nanowire grids. Reducing the deposited sample volume and eliminating the blotting step allows for an advanced degree of control over the thin film formation step and subsequent vitrification quality. Furthermore, this rapid process allows the chameleon to address air water interface (AWI) effects with dispense-to-plunge speeds as fast as 54ms compared to a few seconds with previous generation commercial instrumentation.

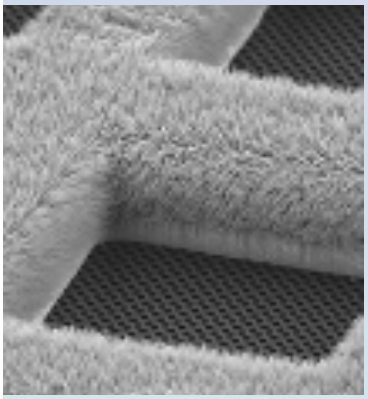
View chameleon video **here**



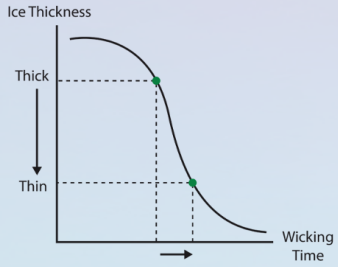
The chameleon instrument deck



An image from the onboard chameleon camera showing the picolitre dispenser setup



Scanning electron microscope image of a self-wicking nanowire grid



Intelligent software guides the user through the wicking characterization step

Standardize high quality specimen amenable for data collection

The one size fits all approach of traditional blot and plunge freeze methods does little to address sample specific characteristics and reduce variability. The resulting inconsistent ice quality and unmitigated air water interface effects lead to unnecessary screening along with experimental repetition with untraceable results. Incorporating chameleon into the cryo-EM workflow represents a paradigm shift in how we approach the bottleneck of specimen optimization and screening.

To reduce the number of research cycles necessary to define a clear path to success, the first step should be to achieve reproducible high-quality specimen such that sample behavior can be characterized in the same initial screening session. The chameleon reduces the screening load on cryo-EM infrastructure by allowing researchers to control and then screen for film thickness using onboard cameras during sample vitrification. During the expensive cryo-EM screening step, loading specimen with only good ice can further reduce costs associated with the time-consuming process of searching many grids with inconsistent non-uniform ice quality on each.

Adjust plunge speed for unique sample behavior

Through the benefit of advanced instrumentation and methods, we now know AWI effects to be ubiquitous and sample specific. It has been shown that addressing the rate of particle adsorption to the air water interface can have beneficial effects, improving data quality and therefore reducing the amount necessary to achieve high-resolution reconstructions. The chameleon can produce multiple vitrified grids in a single session at different dispense to plunge times resulting in a set of specimens to screen for unique sample behavior related to the rate of adsorption to the AWI.

Once information is gained about resulting concentrations of particles amenable for data collection and rates of air water interface effects, subsequent chameleon sessions can be customized and directed to mitigate the unique sample behavior by either plunging faster or slower.

Future: Sample specific vitrification workflows

Customized vitrification protocols based on unique sample behavior can build on an expanding knowledge base emerging from the cryo-EM field to further eliminate the sample optimization bottleneck. As an increasing number of new samples are run on chameleon we can learn about shared sample characteristics with regards to air water interface effects and categorize behavior towards customized vitrification protocols. This shift in thinking represents a transformative first step to allowing sample specific workflows for cryo-EM preparation optimization.

The emerging published results from chameleon have shown that reproducibility and improved sample quality translates to cost savings across the entire cryo-EM workflow. Whether you are just getting started in cryo-EM or looking to solve a particularly difficult sample, at SPT Labtech we have an expert team of scientists with years of experience in the cryo-EM field ready to lend a hand. Contact the SPT Labtech team today to learn more about the chameleon cryo-EM workflow.

Reference List

D.S. Booth, A. Avila-Sakar, Y. Cheng, Visualizing proteins and macromolecular complexes by negative stain EM: from grid preparation to image acquisition doi: 10.3791/3227 Published: December 22, 2011

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Part 4: Towards Routine Sample Specific Optimization for All

Existing Challenges

As cryo-EM adoption has grown so has the demand to prepare specimen using conventional commercial instruments and DIY set ups. However, most samples exhibit non-ideal behavior and the resulting specimen rarely achieve the reproducible and traceable consistency needed to drive optimization towards improved outcomes for detailed structure determination. The need to routinely optimize for sample specific behavior for improved specimen quality is widely recognized as a key unresolved step in the workflow and restricts the use of cryo-EM in novice hands.

Conventional sample optimization for cryo-EM utilizes multiple instruments, consumables originating from various manufacturers and user dependent experimental methods to deal with the detrimental effects brought about by protein interactions with the air-water interface. There are not yet any optimization workflows specific to sample type. Only a consensus that optimization is required for most samples and that approximately ten different methods, each with an optimization routine of their own, will be attempted in combination before improvement is seen. Additionally, none of the currently available methods can be determined to work in advance of sample preparation.

An abundance of low-quality samples causes downstream inefficiencies in the workflow. Bottlenecked microscope and computing resources and increasing costs (time and money) result in a failure to achieve early research milestones important for funding and resource allocations.

Optimizing Vitrification for Your Sample-Specific Behavior

With the introduction of next generation sample preparation technology realized through controllable self-wicking nanowire grids and chameleon, users can target discrete fast plunge times and optimal film thickness for the sample at hand. Implementing automation and outcome-based workflows makes sample-specific optimization accessible and routine.

A common effect of conventional slow (>1 sec) blotting or pin printing techniques is up to a 30-fold increase in sample concentration at the air-water-interface. The resulting interactions lead to denaturation, which is pronounced for less stable proteins, and can result in limited particle orientations and distributions necessary for structure determination. Low sample quality results in limited achievable resolutions and the need to collect extremely large data sets with a very low percentage of particles remaining in the resulting reconstructed maps.

A known feature of high-speed spraying devices is reduced particle adherence at the air water interface. Therefore, the observable concentrated particle density at the air water interface decreases when applying faster dispense-to-plunge times for a sample at a given concentration. The effect is non-linear and likely results from a combination of molecular characteristics that influence the rate of adsorption along with the subsequent formation of a denatured protein network or film with secondary characteristics. The relationships are sample dependent and early use suggests an understanding of this effect for each sample is critical to determining an optimization pathway towards improved sample quality for high resolution.

Rather than eliminate all interactions between protein and the air water interface, since it is impractical to outrun protein adsorption entirely, routine chameleon protocols target a range of discrete fast dispense-to-plunge times to initially characterize sample behavior with regards to concentration, wicking speed, film thickness and observable particle density and then optimize based on outcomes.

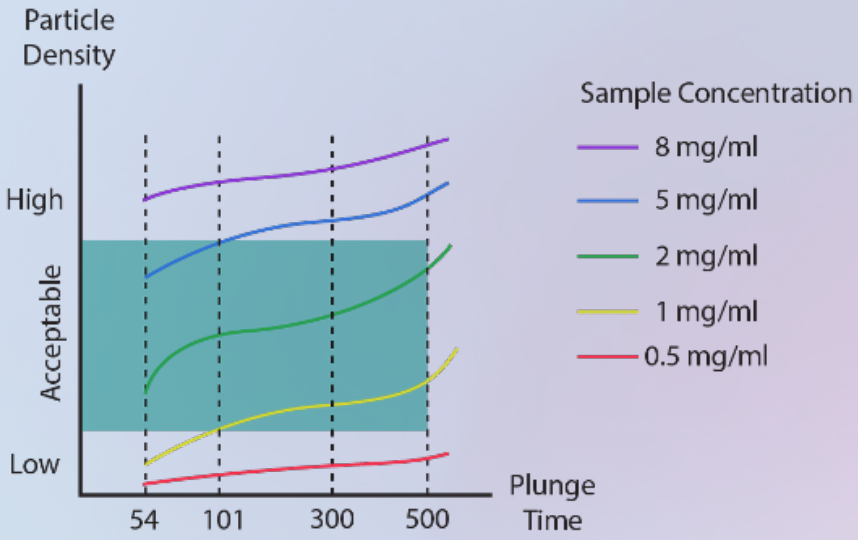

Fig. 1 Examples of available dispense-to-plunge times for sample-specific behavior with regards to starting sample concentration and resulting particle density

How Do I Make chameleon Work for Me?

Approaching the chameleon with a new sample requires a first step to determine the fastest plunge time possible for a given sample concentration without particle density dropping too low. Using the sample concentration in hand the system is directed to prepare a few grids at a range of reducing plunge times. Typically, the plunge time range will start at > 500ms for observable particle densities comparable to conventional methods. Early results show improved quality at a range of sample dependent plunge times (1000ms – 54ms) for a variety of samples. The starting plunge time of ~500ms, slow by chameleon standards, represents a 6-10x reduction in dwell time compared to other commercially available sample preparation instruments. Therefore, the evaluation of 'slow' and 'fast' plunge times along with film thickness from 'good' to 'overwicked' is necessary to determine optimal conditions for the sample at hand.

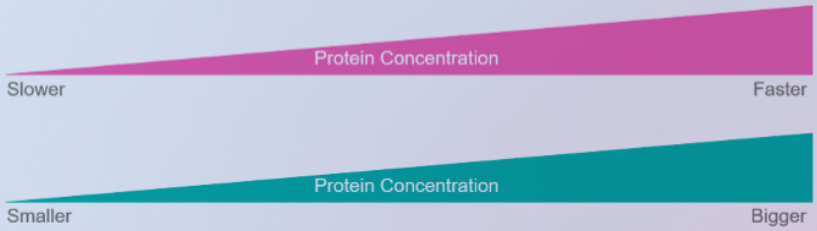

Fig.2 Concentration and specimen size will determine what dispense-to-plunge times are appropriate

After gaining an understanding of the appropriate plunge time range and ice thickness for the sample concentration and characteristic behavior, a second freezing session with a set of much more stringent acceptance criteria corresponding to a narrow range of plunge times and film thickness can produce multiple examples with consistent outcomes.

What Results Can Be Achieved?

Results point to improvements in specimen quality related to consistent and controlled film thickness, denaturation, and preferred orientation for a variety of samples across a range of sample-specific plunge times from fast (54ms) to slow (>500ms).

Table 1. Recent sampling of chameleon solved structures exhibiting improved quality related to thin film formation and thickness, denaturation, and preferential orientation.

Structure	Size(kDa)	Resolution (Å)	Publication
DNA Polymerase Polζ-DNA-dCTP Ternary Complex	258	3.1	Nature Structure & Molecular Biology 2020
Aβ-bound Presequence protease	117	3.3	Research Square Preprint 2020
SARS-CoV2 Spike Protein/ Nanobody Complex (DLS)	382	3.7	Nature Structure & Molecular Biology 2020

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1. B. Carragher, Y. Cheng, A. Frost, R.M. Glaeser, G.C. Lander, E. Nogales, H.-W. Wang, Current outcomes when optimizing 'standard' sample preparation for single-particle cryo-EM doi:10.1111/jmi.12834 Published: September 19, 2019

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A Paradigm Shift Towards the Future

The routine optimization protocol available through chameleon represents a paradigm shift by addressing the unique sample dependent air-water interface effects by adjusting the vitrification step to the sample behavior instead of requiring researchers to introduce additional methods and optimization cycles to modify sample behavior to accommodate standardized plunge freezing devices. To be able to capture biochemistry consistently and confidently researchers need the ability to freeze where and when the biochemistry is carried out, placing the requirement for change squarely on the sample preparation devices to modernize, improve ease-of-use, and generate high quality outcomes efficiently to achieve early milestones routinely.

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