Ultrastructural Studies by Correlative Stochastic Optical Reconstruction Microscopy and Electron Microscopy

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Ultrastructural Studies by Correlative Stochastic Optical Reconstruction Microscopy and Electron Microscopy

A dissertation presented

by

Doory Kim

to

The Department of Chemistry and Chemical Biology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Chemistry

Harvard University

Cambridge, Massachusetts

April, 2015
Ultrastructural Studies by Correlative Stochastic Optical Reconstruction Microscopy and Electron Microscopy

Abstract

Fluorescence light microscopy (LM) and electron microscopy (EM) are two of the most widely used imaging modalities for probing cellular structures. In this dissertation I present our works in both developing methods of several correlative super-resolution fluorescence light microscopy (LM) and electron microscopy (EM) assays by combining stochastic optical reconstruction microscopy (STORM), a super-resolution imaging technique with several different EM imaging modalities and applying super-resolution microscopy to investigate the distributions and interactions of purine biosynthetic enzymes organization complex called purinosomes within the cell.

The first work contained in this dissertation is to develop Correlative fluorescence light microscopy and electron microscopy allows the imaging of spatial distributions of specific biomolecules in the context of cellular ultrastructure. Recent development of super-resolution fluorescence microscopy allows the location of molecules to be determined with nanometer-scale spatial resolution. However, correlative
super-resolution fluorescence microscopy and electron microscopy (EM) still remains challenging because the optimal specimen preparation and imaging conditions for super-resolution fluorescence microscopy and EM are often not compatible. Here, we have developed several experiment protocols for correlative stochastic optical reconstruction microscopy (STORM) and EM methods, both for un-embedded samples by applying EM-specific sample preparations after STORM imaging and for embedded and sectioned samples by optimizing the fluorescence under EM fixation, staining and embedding conditions. We demonstrated these methods using a variety of cellular targets.

In the second part of this dissertation, I focus on the study of dynamic purine biosynthetic enzymes organization complex called purinosomes. Purine biosynthetic enzymes are assembled into dynamic multi-enzyme complex called purinosomes. However, spatial or temporal control of these structures remains unknown. Here, we explored the endogenous purinosomes in medically important HGPRT-deficient LND fibroblasts in order to understand the de novo purine biosynthesis. Using super-resolution microscopy we investigated the interaction of purinosomes and mitochondria or microtubules using photoactivatable fluorescent protein, mMaple3 and LND fibroblast as an ideal model system for the endogenous purinosomes formation in order to avoid possible protein aggregation problems. The STORM images with this ideal model system revealed a highly correlated spatial distribution of endogenous purinosomes with mitochondria or microtubules, suggesting direct physical associations between two structures. In addition to identifying endogenous purinosome association with other cellular components, we also demonstrated that mTOR directly influenced the purinosome association with mitochondria. Inhibition of mTOR decouples spatial
correlation of purinosomes with mitochondria. These data provide strong evidences for physical and functional association of endogenous purinosomes with mitochondria and microtubules.
Acknowledgements

First and foremost, I would like to express my sincere gratitude to my advisor Professor Xiaowei Zhuang for guiding and supporting me over the years. She dedicated her time and effort to help me overcome many crisis situations and encourage me to continue my research. I could not have imagined having a better advisor and mentor for my Ph.D study. I feel honored to have had a chance to work with her and learn from her. Besides my advisor, I would like to thank the other members of my committee, Professor Jeff Lichtman, Professor Adam Cohen and Professor Max Nibert for their insightful comments and the assistance they provided at all levels of the research project. I also owe my deepest gratitude to my Master’s advisor, Professor Seong Keun Kim at Seoul National University for guiding me through the exciting world of scientific research. He has constantly provided me with mentoring, support, and encouragement throughout graduate school and I have learned so much about how to be a strong and good scientist from him.

It has been a pleasure to work with and learn from a group of talented and gifted people in the Zhuang lab. I thank Dr. Miriam Bujny for training me early on in the experimental techniques; Dr. Sara Jones for helping me working on the projects of purinosome and sharing her experiences; Dr. Sang-Hee Shim, Dr. Joshua Vaughan, Dr. Shu Jia and Dr. Ruobo Zhou for their scientific discussions and continuous encouragement; Dr. Eileen Sun and Jiang He for their suggestions on the virus project; Dr. Ke Xu and Dr. Hazen Babcock for teaching me all the skills I needed to do dual-
objective STORM experiments; Dr. Yari Sigal and Dr. Colenso Speer for discussions regarding the resin-embedding work; our supportive CCB friends, Boran Han, Guiping Wang, Kok Hao Chen, George Hao and Chenglong Xia for their friendship and encouragement; and Matt Kilroy and Alec Goodman for their administrative help. I also would like to thank all of other Zhuang lab members past and present for their sustained support, help and useful discussions.

I have also been fortunate to work with incredibly talented collaborators outside of the lab. I am grateful to my collaborators, Professor Mark Ellisman, Professor Stephen Benkovic, Professor Xin Zhang, Tom Deerinck, Keith Chan and Rongmei Qu for sharing expertise, insightful comments and suggestions. In the various laboratories I have been aided for many years in using electron microscopy by many people. In particular, I want to thank Richard Schalek, Nicholas Antoniou and Maria Ericsson for their technical assistances and useful guidance in the use of EM-related setups.

I am deeply thankful to my family for their unlimited love, support, and constant encouragement I have gotten over the years. I would especially like to thank my mom for always listening to me, understanding me and never complaining. I undoubtedly could not have done this without her. I am also grateful to my dad both for being my wonderful role model and giving me insightful career advice. I would like to dedicate this thesis to my loving parents. Also I thank my two elder sisters and younger brother: Asa Kim, Sori Kim and Han-eol Kim. They were always supporting me and cheering me up with their best wishes.

Last but not the least, I would like to thank my friends. I would especially like to
mention my beloved friends: my longtime best friend, Jiwon Lee for always caring about me and encouraging me; Yoonsuk Hyun for giving me moral support and a lot of happy memories; my longtime friend, Junghye Han for always cheering me up; my longtime roommate, NaNa Keum for the years of sharing life together; Hsiao-Han Chang for listening to my stories and sharing happy moments during the course of my graduate studies.

My sincere thanks also goes to my friends in the chemistry department: Sangjin Kim, Hong Geun Lee, Jiwon Kim, Jeong-Mo Choi, Heejun Lee, Bon Jun Koo, Wooyoung Hong, Sangwoo Shim, Seokhee Kim, Myung-Han Yoon, Shasha Chong and Sirui Zou at Harvard University; Woonju Song, Sarah Yunmi Lee and Hongik Hwang at MIT; Youngbin Lim, Sanghak Lee, Eunsang Lee and Jinyoung Chang at Seoul national University. I also would like to thank my Catholic friends from Parish of St. Paul in Harvard Square, Eunjoo Park, Sapyung Shin, Jinhyung Kim, Sungchun No, Jongho Lee, Jeehye Kim and Won Kim, who have brought encouragement and fun to my life during graduate school.
This work is dedicated to my parents, Chang-Hong Kim and Injung Chang.
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Chapter 1. Introduction

1.1. Introduction

Fluorescence light microscopy (LM) and electron microscopy (EM) are two of the most widely used imaging modalities for probing cellular structures. These modalities have distinct strengths and weaknesses that complement each other. Fluorescence LM allows spatiotemporal localization of labeled biomolecules, such as proteins and nucleic acids, with high molecular specificity and sensitivity. Multi-color imaging using spectrally distinct fluorescent labels allows several molecular targets to be imaged simultaneously and their interactions to be directly probed. Recently, various super-resolution fluorescence imaging techniques have been developed to substantially surpass the diffraction limit, allowing molecular structures in cells to be imaged with nanometer-scale resolution[1-3]. Transmission and scanning EM methods provide higher image resolution than light microscopy, including super-resolution fluorescence microscopy. Under standard staining or contrasting conditions, EM also allows, subcellular compartments, including lipid bilayer-based membranes, to be imaged with ultra-high resolution. A number of specific molecular structures can also be identified from EM images based on characteristic shapes, but such molecular structures are relatively few compared to the great diversity of macromolecules known to be made and employed to form complexes, organelles, cells and tissues. Molecule-specific imaging using EM traditionally involves immunolabeling with ferritin or gold conjugates, which contribute to low labeling efficiencies[4,5]. Several approaches have been recently developed to use
genetically encoded tags, such as the tetracysteine biarsenical system[6,7], miniSOG[8] and APEX2[9,10], to provide EM contrast for specific molecules. These approaches give much higher labeling efficiency than immunogold. However, “multi-color” EM imaging of more than one or two molecular targets still remains challenging. It is thus desirable to perform correlative fluorescence LM and EM such that the spatial locations of specific molecules can be determined using fluorescence imaging in the context of the cellular ultrastructure revealed by EM.
1.2. STORM

1.2.1. Introduction

Fluorescence light microscopy is a widely used technique in molecular and cell biology for studying protein localization, interaction and function within the cellular context with high molecular specificity. However, a lot of sub-cellular structure can't be readily resolved by conventional fluorescence light microscopy because of diffraction limit, about 200-300nm in the lateral direction and 500-700nm in the axial direction. The diffraction limit can be explained by the full width half maximum (FWHM) of the point spread function (PSF) from a single point emitter[11].

\[
\text{FWHM (x, y)} = \frac{0.61\lambda}{\text{N.A.}}
\]

\[
\text{FWHM (z)} = \frac{2\lambda\eta}{\text{N.A.}^2}
\]

where \(\lambda\) is the wavelength of the emitted light, N.A. is the numerical aperture of the objective lens, and \(\eta\) is the index of refraction of the medium. If two point sources are distant from each other, they are well resolved. However, if they are closer than the FWHM of the PSF, their PSFs will appear overlapped and not be resolved.

In order to substantially overcome this limit, various super-resolution fluorescence imaging techniques have recently developed rapidly[12-17]. There are roughly three different categories in these methods: (1) the stochastic super-resolution methods based on the detection and localization of single fluorescence molecule in high precision, such as stochastic optical reconstruction microscopy (STORM) [12] or (fluorescence) photoactivation localization microscopy ((F)PALM)[13,14], (2) the deterministic super-resolution methods using the PSF modification, such as stimulated emission
depletion(STED) microscopy[15] or saturated structured-illumination microscopy (SSIM) [16], and (3) the very recently developed scalable super-resolution method based on the idea of making objects larger to make them more visible, such as expansion microscopy[17]. With the development of super-resolution fluorescence microscopy technologies, the location and composition of proteins in a cellular event could be determined precisely and many previously unanswerable questions could be addressed. Here, we focus on the first approach, STORM and discuss its principle and advances.

STORM relies on the high precision localization of individual fluorescent molecules by isolating them from the adjacent fluorescent molecules[12]. The PSFs from the closely localized fluorescent molecules which could be possibly overlapped to each other can be separately detected by controlling them to emit the fluorescence at the different time points (different frames in a movie). The photoswitchable probes which can be optically switched between a fluorescence and a dark state are used for this stochastic activation, including organic dyes and fluorescent proteins[18]. For example, cyanine dyes can be switched on and off reversibly upon activation illumination and deactivation illumination at different wavelengths in the presence of thiol buffer and it has been shown that cyanine dyes proceed through the dark state by forming an adduct with a deprotonated thiol[19]. Taking advantage of photoswitching property, only a subset of the well-isolated fluorescent molecules is imaged at each time point and their centroid positions can be fit in high-precision. By iterating this process until most or all of molecules are imaged and superimposing them, the image of structures can be reconstructed in high-resolution.

Spatial resolution of STORM is highly related to the localization precision
although a number of other factors can also affect to the resolution, including labeling density, switching properties, sample drift and data analysis[20-22]. The localization precision is known to proportional to the photon number detected per switching event[23]. The underlying idea is that the localization of single molecule is determined by the detection of multiple photons emitted from a fluorophore. Therefore, each photon detected can be thought of as individual measurement of the position of single fluorophore and uncertainty in the position is inversely proportional to the number of measurement, photons, in a similar way to the relation between the standard error and the sample size in statistic. Given that the fluorophore emitting high photon numbers give high localization precision, the brighter fluorophores are preferable.

As STORM is an extended conventional fluorescence microscopy, it has been possible to add capabilities such as multi-color imaging, three-dimensional(3D) imaging, and live-cell imaging, similar to the way conventional fluorescence microscopy has been extended[21,24,25].

1.2.2. Multi-color STORM

Multi-color imaging is an important capability of fluorescence microscopy since it can be used to map interactions between different biological structures. Taking advantage of high-resolution of STORM, multi-color STORM can be more informative because it can give the colocalization information in high accuracy. In a similar way to the multi-color conventional imaging, various combinations of the photoswitchable dyes and
fluorescent proteins can be used for multi-color STORM[24]. In addition to using emission color palette, STORM is also able to use activation color palette. The cyanine dyes (‘reporter’) can be selectively activated by paring them with different ‘activator’ dyes which facilitate reactivation of the reporter in a pair. By pairing different activators with a same kind of reporter and illuminating activator’s corresponding excitation wavelength, different pairs of probes can be activated differently and finally be differentiated. Therefore, combinatorial pairing reporters and activators in multi-color STORM enable more various multicolor palettes and visualization of molecular interactions of more targets in high resolution.

1.2.3. 3D STORM

As the most cellular structures exist in three-dimensional space, it is more informative to learn three-dimensional structures in biological system. In 3D STORM, the lateral positions can be determined from the centroid of its image in a same way with 2D STORM, and the axial position is determined by ellipticity of its image using the astigmatism approach[25]. By inserting the cylindrical lens into the imaging path, different focus for x and y direction are created, generating ellipticity of its image. Because the ellipticity is determined by axial (z) position, it can be used to derive the z value in high precision by comparing the widths in x- and y-directions(ellipticity) to a calibration curve generated from molecules at known z-positions. With this approach, the lateral resolution of 20 nm and axial resolution of 50nm (full width at half maximum, FWHM) have been achieved[25].
1.2.4. Dual-objective STORM

Further development of STORM has continued to improve its power and versatility\cite{21,26,27}. One of them is dual objective STORM which combines astigmatism imaging, dual-objective detection and small-molecule labeling in STORM to improve its resolutions\cite{26}. By sandwiching the sample between two opposing objective lenses, the total photon numbers detected can be doubled, improving the image resolution by 1.4-fold. This approach has been experimentally demonstrated with the actin cytoskeleton in mammalian cells and $<10$nm resolution in the lateral direction and $<20$nm resolution in the axial direction have been achieved\cite{26,27}.
1.3. Electron Microscopy

1.3.1. Introduction

The electron microscopy is a type of microscopy that uses accelerated electrons as a source of illumination to create an image of the specimen. Electrons have characteristics of both particles and waves as light does. Accelerated electrons in vacuum can be straight line like light and its path can be shaped by electric and magnetic fields similar to the way glass lenses are used to focus and guide light path. As previously stated, the resolution of a microscope depends on wavelength of the illumination source(λ) and the numerical aperture of the lens(N.A) [11].

\[
\text{limit of resolution} = \frac{0.61\lambda}{\text{N.A.}}
\]

Because of wave-like behavior of an electron, the wavelength of electron beam can be determined by accelerating voltage which is a potential difference of voltage between the filament and the anode.

\[
\lambda = \frac{\hbar}{\sqrt{2meV}} = \frac{1.5}{\sqrt{V}} \quad \text{[nm]}
\]

where \( h \) is the Plank’s constant, \( m \) is electron mass, \( e \) is electronic charge and \( V \) is accelerating voltage. By applying high accelerating voltage(~50kV), the wavelength of an electron beam(~0.0054nm) can be about 100,000 times shorter than that of visible light, and hence electron microscopy can achieve higher resolution than light microscopy.

Accelerated electrons can behave like light only in vacuum. There are several reasons why vacuum is required for the proper operation of electron microscopy. First, hot filament, which is used as the electron sources, oxidize and burn out in the presence
of air molecules at atmospheric pressure. Secondly, air particles and dust could block and interfere the electron beam before it reaches the sample. Thirdly, electric and magnetic optics in electron column can be operated properly in vacuum[28]. Therefore, it should be operated in a vacuum of at least $10^{-5}$ torr and separate pumps and airlocks are used for quick and efficient vacuum under automatic control[29].

The requirement of vacuum in electron microscopy limits the specimen condition. The specimen should be clean, dry and vacuum-compatible, and any volatile materials should be removed by drying or freezing. It also should not be altered by electron bombardment. For example, non-conductive component could cause charging artifact by accumulating charge under electron bombardment. In order to prevent charging artifact, non-conductive specimen should be coated by a conductive layer, such as gold, palladium or carbon. The coating should be thin enough to allow the interaction of electrons and specimen underneath the coat layer and thick enough to provide continuous conductive film. However, some of recently developed electron microscopy techniques have expanded the type of samples that can be examined. For example, environment SEM allows researchers to observe specimens in a broader range of conditions, such as wet and non-conductive specimens with a specially designed vacuum system. And Cryo-electron microscopy allows researchers to investigate bio- or organic materials in their natural context with a freezing technique[30,31].

Electron microscopy provides different types of information by analyzing the different kinds of interactions of electromagnetic waves and a specimen and it is being applied successfully in a wide range of life sciences, material sciences and electronics. Also several types of electron microscopy have been developed to investigate different
aspects of a sample. Among them, scanning electron microscopy and transmission microscopy will be discussed here as the most commonly used electron microscopy techniques.

1.3.2. Transmission Electron Microscopy

In TEM, the electron beam passes through the specimen and carries information about the structure of the specimen (Figure 1.1). Therefore, the specimen needs to be thin enough to be electron transparent and stable enough to preserve its atomic structure under the electron beam penetration process. The sample also should be small enough to be introduced in the electron column owing to the small size of the sample holder (~3mm in diameter). Most of biological samples are nearly electron transparent because they consist of light elements (C, O, H, N, S, P and etc.) and thereby they must be stained with heavy metal salts and sliced into very thin sections to achieve the best results during examination in the TEM. The TEM sample preparation in this case includes fixation, dehydration, embedding, ultra-microtomy and staining of the section.

There are five main components of TEM: an electron source, electromagnetic lens, a specimen stage, a detector and vacuum system. In TEM, accelerated electrons are generated from the electron gun and concentrated onto the specimen by condenser lens in a similar way to light microscopy. The electron beam, which passed through the specimen, is focused by objective lenses and magnified by projector lens until it is recorded by hitting a detector.
The electron source can be chosen depending on the applications. Thermionic sources, which expels electrons by applying high temperature using thermionic emission, such as tungsten or LaB$_6$, is generally cheap, but has relatively low brightness and limited lifetime. Field emission gun (FEG), which emits electron through tunneling effect using...
high electric field, is expensive but has the highest imaging and analytical performance because of high brightness[32].

In the optics of TEM, the magnetic field generated by the current flowing through a coil determines the performance of the lenses[32]. They are similar to that of light microscopy in terms of their functions. The condenser lenses converge the electron beam to a focal point and hence they determine the intensity of electron beam[32]. The objective lens is used to focus the electron beam and initially magnify the image and the projector lenses further magnify the image until it is projected on to the phosphorescent screen. In order to reduce and exclude extraneous electrons in the electron beam path, several apertures are used to determine the diameter and the spot size of the electron beam at the specimen which determines the resolution. The resolution can be improved with small aperture size by reducing the spot size[32].

In order to generate a final image, the electron beam is projected onto the phosphorescence screen and a film camera, or a charge-coupled device (CCD) camera.

### 1.3.3. Scanning Electron Microscopy

In SEM, the finely focused electron beam is scanned in a raster fashion over a rectangular area of the sample and scattered electrons caused by the interaction of the incident electron beam with atoms in the sample are detected above the specimen. Therefore, sample preparation of thin sectioning is generally not required in SEM as the electrons don't need to pass through the sample. In general, SEM has a larger sample holder than TEM allowing investigation of bulk samples up to many centimetres in size depending on instrument design and setting.
SEM, like TEM, consists of five main components: an electron source, electromagnetic lens, a specimen stage, detectors and vacuum system. As the lenses are positioned above the specimen, they resemble the upper portion of TEM and the length of electron column is shorter than that of TEM (Figure 1.2).

![Figure 1.2. Comparison of the light microscope with TEM and SEM](image)

(A) Main components of light microscope (B) Main components of TEM (C) Main components of SEM.

The use of different detectors in SEM allows the various signals to be detected. Different types of signals originate from the different volumes of interactions from which each signal is produced. There are main three signals measured in SEM providing different information about the sample: secondary electrons (SE), back-scattered
electrons (BSE) and X-rays (Figure 1.3). First, SE are generated from a small volume (<10nm) around the point where the incident electrons hit the sample in an inelastic fashion (loss of energy) [33]. The incident electrons lose the kinetic energy and it is used for the secondary electrons of an atom to leave from the surface of sample (binding energy) and for their small kinetic energy. SE is the most frequently used signals in SEM and it is usually used for the sample's surface topography because of its topographical contrast.

![Figure 1.3. Signals from electron beam-specimen interaction and their interaction volumes.](image)

Secondly, BSE are generated from deeper parts of the sample volume than SE by the elastic collision of an incident electron with a nucleus of a sample. They can leave the surface of the sample with negligible kinetic energy loss taken by a sample because of
negligible mass of electrons. The intensity of BSE varies with atomic number since heavy atoms with a high atomic number are much stronger scatters of electrons than light atoms. Therefore, BSE imaging provides compositional information from the atomic number contrast. One application of BSE imaging based on this is the thin section of biological samples[34]. The BSE images of plastic-embedded, sectioned and heavy metal stained biological specimens closely resemble the conventional TEM images.

Thirdly, X-rays are emitted from the deepest part of a sample when the electron of inner orbital of a sample atom is vacant and the vacant inner shell is filled by the electron from higher energy orbital. As the wavelength of X-ray is the energy difference between these two orbitals, it is characteristic and provides the information of elemental composition. In order to analyze the X-rays signal, energy-dispersive(EDXS) and wavelength-dispersive(WDXS) spectrometers can be installed in SEM. These diverse SEM analyses from various signals have become invaluable in a variety of science and industry applications.
1.4. Outlines of this thesis

The work contained in this dissertation is to develop methods of several correlative super-resolution fluorescence light microscopy (LM) and electron microscopy (EM) assays for investigating the protein distributions and ultrastructures of cells and to study the protein associations in a cell using super-resolution fluorescence light microscopy. Chapter 1 provides an overview of correlation light and electron microscopy. It is meant to discuss the significance of correlative microscopy. Also it describes the basic background of STORM and EM techniques which are used in our work. Chapter 2.1 specifically discusses the recent development of correlative super-resolution microscopy and electron microscopy as an introduction. With this introduction in place, Chapters 2.2 describes the Correlative 3D STORM and scanning EM (SEM) and Chapter 2.3 describes the Correlative 3D STORM and transmission EM (TEM) for unembedded samples. In these methods, we performed all EM-related sample treatments, which could affect the brightness and photoswitching properties of dyes, after STORM imaging, hence both STORM and EM imaging conditions could be separately optimized. Chapter 2.4 presents the Correlative STORM and back scattered electron (BSE)-SEM imaging of embedded and sectioned samples. In this chapter, we compared various embedding materials and conditions, fixatives, and negative staining agents to find near optimal conditions for both STORM and EM imaging. We applied these protocols to several cellular and viral structures in Chapter 2. Chapter 2.5 overviews the projects described in this thesis and contains the detail methods. Chapter 3 describes the study of purine biosynthetic enzymes organization called purinosomes. In this chapter, we demonstrated
that physical association of endogenous purinosomes with mitochondria and microtubules using multi-color STORM, mMape3 and LND cells in order to understand the spatial control of these structures.
Chapter 2. Correlative Stochastic Optical Reconstruction Microscopy and Electron Microscopy

2.1. Introduction

Correlative fluorescence LM and EM has been reported for both conventional[35-38] and super-resolution fluorescence imaging[13,39-48]. In particular, correlative super-resolution LM and EM methods have been reported for unembedded samples using negative staining[13,40,42,45,48] or platinum replica for EM contrast[41,44], in plastic-embedded samples using negative staining for EM contrast[39,43,46], and in cryoEM samples without any metal staining[47]. Although these studies have demonstrated the impressive power of correlative microscopy, it remains challenging to obtain optimal quality for both EM and super-resolution LM images in correlative microscopy. For example, the light emission from fluorescent proteins and dyes can be substantially compromised in the acidic and oxidizing conditions typically used for plastic embedding protocols. Osmium tetroxide, which is often used as a strong fixative and stain for membrane structures in EM, can severely quench the fluorescence signal and hence an extremely low concentration of osmium tetroxide (0.001% OsO$_4$ and 0.1% KMnO$_4$) has been used to minimize the photon loss of fluorescent proteins in correlative super-resolution imaging[39,43]. A more recent study
uses organic fluorophores for super-resolution imaging and another weak fixative and stain, uranyl acetate, for EM contrast[46]. These relatively mild fixatives and stains compromise the ultrastructure preservation and membrane contrast in the EM images. Both studies use the acrylic resin as the embedding material[39,43,46], whereas the harder epoxy-based resins are known to be better for ultra-structure preservation and high-quality ultrathin sectioning[39]. Moreover, since photoswitching of the dye molecules required for super-resolution imaging is partially inhibited when the dyes are embedded in resins, the quality of super-resolution images is also compromised by the resin embedment[46]. Correlative super-resolution fluorescence and SEM/platinum-replica TEM studies[41,44,45,48] circumvent the above problems by performing fluorescence imaging before staining for EM, but these approaches can only be applied to imaging structures relatively close to the cell/sample surface. Correlative imaging with cryo-electron tomography[47] does not require any fixation or staining for EM contrast, but the long-working distance objective (NA ≈ 0.7) required for imaging samples in the cryo chamber has a relatively low photon collection efficiency, giving lower fluorescence resolution. Thus, additional method development is needed to complement the above approaches and optimize the power of correlative super-resolution fluorescence and electron microscopy.

Here, we present several correlative super-resolution fluorescence and electron microscopy assays by combining three-dimensional (3D) stochastic optical reconstruction microscopy (STORM)[2,12,25] with several EM imaging modes. These include protocols for imaging unembedded samples and protocols for imaging embedded and sectioned samples. In the former case, we performed all EM-related sample treatments, which
could affect the brightness and photoswitching properties of dyes, after STORM imaging, hence both STORM and EM imaging conditions could be separately optimized. For the embedded and sectioned samples, we compared various embedding materials and conditions, fixatives, and negative staining agents to find near optimal conditions for both STORM and EM imaging. We applied these protocols to several cellular and viral structures.
2.2. Correlative 3D STORM and scanning EM (SEM) for un-sectioned samples

2.2.1. Correlative 3D STORM and scanning EM (SEM) for unembedded samples

We first present a simple correlative 3D STORM and SEM imaging method for hydrated, unembedded samples. In this method, most of sample preparations for either STORM or SEM were not changed from each respective protocol used for each imaging modality. The STORM imaging was performed prior to any sample preparation required for EM imaging. Therefore, as the fluorescence signal acquisition precedes the addition of any EM fixative or stains, the optimal fixative and stain could be used for EM, and hence neither STORM nor EM images were substantially compromised. The only minor change was the glass substrate used -- to find the same regions of interest (ROI) in the STORM and EM setups, we used photo-etched gridded coverslips (No.2 thickness) instead of the No 1.5 glass coverslips optimal for the 1.4 NA oil immersion objective used.

As an initial test of correlative 3D STORM and SEM, we imaged filamentous influenza viruses budding from infected cells. To prepare the sample, we infected human lung epithelial adenocarcinoma A549 cells with a filamentous virus strain (Udorn) for 12 hours and fixed the infected cells with 4% paraformaldehyde (PFA) and 0.1% glutaraldehyde (GA) or Methanol, followed by indirect immuno-labeling of the viral envelope protein HA or the viral matrix protein M1 with primary antibodies and Alexa
Fluor 405 and Alexa Fluor 647 co-labeled secondary antibodies. Here, Alexa 647 serves as the photoswitchable dye and Alexa 405 facilitate the activation of Alexa 647 with a low power 405 nm light\cite{24} (Figure 2.2.1A,B).

**Figure 2.2.1. Correlative 3D STORM and SEM imaging.** (A) Flowchart of the major steps in the correlative 3D STORM and SEM imaging of unembedded samples. (B) Schematic overview of the correlative 3D STORM and SEM
We first obtained 3D STORM images of the virus filaments with an optimal No. 1.5 coverslip to obtain a high quality STORM image without any concern of EM (Figure.2.2.2A-C,E). The 3D STORM image of the virus filament (Figure.2.2.2A,C, left) and its xy cross-section (Figure. 2.2.2A,C, right) revealed the hollow shape of the filamentous and spherical virions that are difficult to resolve by conventional fluorescence microscopy. The transverse profile of the xy cross-section revealed a filament width of 90 nm(Figure. 2.2.2D), which agrees with the known diameter of a virus filament (80 - 120 nm)[49]. M1 matrix layer is located beneath the lipid membrane and hence its transverse profile of the xy cross-section revealed narrower width of 50nm(Figure.2.2.2E-G), which also agrees with the known diameter of M1 matrix protein layer[50].
Figure 2.2.2. 3D STORM imaging of individual influenza viruses. (A) Schematic representation of spherical and filamentous influenza virus and internal composition (HA and M1). (B) Left: 3D STORM image of a spherical Udorn virus immuno-labeled for HA. Right: xy cross-section of the 3D STORM image of a spherical Udorn virus in the same region. (C) Left: 3D STORM image of a filamentous Udorn virus immuno-labeled for HA. Right: xy cross-section of the 3D STORM image of a filamentous Udorn virus in the same region. (D) Transverse profiles of localizations corresponding to regions boxed in red in (C). Blue bars: localization frequency measured from the STORM image. (E) 3D STORM image of a filamentous Udorn virus immuno-labeled for M1. (F) Magnified views of the boxed regions in E. (G) Transverse profiles of localizations corresponding to regions boxed in red in (F). Blue bars: localization frequency measured from the STORM image. Scale bars, 100nm (B,F), 500 nm (C,E).
It has been reported that filamentous viruses can bud in bundles overlapping to each other, either in side-by-side or end-to-end arrangement[50]. However, with low resolution imaging, we cannot tell whether those filamentous structures are single budding viruses or virus bundles. In order to test whether STORM imaging is able to resolve those structures, we tried 3D STORM imaging of filamentous Udorn viruses immuno-labeled for M1 for the side-by-side and end-to-end overlapping filaments. The 3D STORM image of the virus filaments (Figure 2.2.3A,C, right) and its xy cross-sections (Figure 2.2.3B) revealed the hollow shape of the filamentous and well resolved closely overlapping filamentous virions that are difficult to be resolved by conventional fluorescence microscopy(Figure 2.2.3A,C, left).
Figure 2.2.3. 3D dual-objective STORM imaging of overlapping filamentous influenza viruses. (A) 3D dual-objective STORM and SEM images of Udorn viruses immuno-labeled for M1 overlapped side-to-side. (B) Transverse profile of localizations corresponding to the region boxed in red in (A). Blue bars: localization frequency measured from the STORM image. (C) 3D dual-objective STORM and SEM images of Udorn viruses immuno-labeled for M1 overlapped end-to-end. Scale bars, 500 nm.
We next prepared the sample in the same way on the photo-etched gridded No. 2 coverslip to perform correlative 3D STORM and SEM (Figure 2.2.4A). The 3D STORM image and the xy cross section could still resolve hollow shape of the virus filament (Figure 2.2.4B,C). The brightness of the Alexa 647 dye (measured as the number of photons detected per switching event) decreased only slightly (by 6%) owing to the use of the thicker photo-etched gridded coverslip as compared to the No. 1.5 coverslip (Figure 2.2.4D). After STORM imaging, the sample was stained with 1% osmium tetroxide, dehydrated in a graded ethanol series, dried by either CO$_2$ critical point drying or transition to hexamethyldisilazane, coated with carbon, and imaged in an SEM using the secondary electron detector imaging mode. The SEM images correlated well with the STORM images with viral filament width measured to be 85nm (FWHM) in the SEM image. In order to estimate how accurate the correlation is, we calculated the normalized cross-correlation between STORM and SEM images and displayed it as a surface plot (Figure 2.2.4E). The cross-correlation map between the STORM and SEM images clearly shows a sharp peak at the center (displacement error of 3.3 nm and FWHM of 33.7 nm). The displacement of the peak from the center indicates the alignment errors between the STORM and EM images and the width of the peak is largely dominated by the feature sizes in the images.
Figure 2.2.4. Correlative 3D STORM and SEM imaging of individual filamentous influenza viruses. (A) Correlative 3D STORM and SEM images of Udorn virus immuno-labeled for HA. Left: STORM image. Right: SEM image. Middle: Overlaid image. (B) Magnified views of the boxed regions in (A). (C) Transverse profiles of localizations corresponding to regions boxed in red in (A). Blue bars: localization frequency measured from the STORM image. Red line: Gaussian fit of the blue bars. (D) Normalized photon numbers per switching event. The results are normalized to the average photon number obtained from samples on the No. 1.5 glass coverslip (photon number = 5233). (E) Cross-correlation between STORM and SEM images. Inset in blue box: Magnified view of cross-correlation. Scale bars, 500 nm in (B,C).
Next, we performed correlative two-color STORM and EM of filamentous virus immuno-stained for both the viral matrix protein M1 (or viral envelope protein HA) and viral ribonucleoproteins (vRNPs). Again, we infected A549 cells with filamentous Udorn virus for 12 hours and fixed with 4% PFA and 0.1% GA, followed by immuno-labeling with M1 (or HA) antibodies and vRNP antibodies, and the Alexa 647-labeled secondary antibodies and Alexa 568-labeled secondary antibodies, respectively. Alexa 568 is also a photoswitchable dye[20]. Previous TEM studies using thin sectioned sample showed that vRNPs are located at the distal end of a budding filamentous virion of relatively short lengths[51]. However it is uncertain whether long filaments also contains only a single set of vRNA at the end or whether there are more vRNPs distributed along the filaments as the full length of the long filaments is not typically contained in a single thin section. Correlative 3D STORM and SEM doesn't require sectioning, allowing us to visualize the whole length of the filaments (Figure 2.2.5A-E).
Figure 2.2.5. Correlative two-color STORM and SEM imaging of virus filaments and their vRNPs. (A) Correlative STORM and SEM images of budding Udorn virus filaments immuno-labeled for M1 (red) and vRNP (green). Left: STORM image. Right: SEM image. Middle: Overlaid image. (B) Magnified views of the boxed regions in (A). (C) Correlative 3D STORM and SEM images of an Udorn virus that does not contain vRNP. (D) Correlative 3D STORM and SEM images of an Udorn virus that shows one vRNP in the virion. (E) Correlative 3D STORM and SEM images of an Udorn that shows two vRNPs inside the virion. The virus is immuno-labeled for HA and vRNP. (F) Distribution of virus filaments depending on the number of vRNPs contained. Scale bars, 5um in (A), 500 nm in (B, C) and 100 nm in (D, E).
From 69 released filamentous virions, we observed that 56% of the virions did not contain vRNPs, 36% had one vRNP at the end, and 8% showed two or more vRNP spots (Figure 2.2.5F). Therefore, correlative images showed that the majority of budding viruses have vRNPs located at the distal end, consistent with a previous cryo-EM observation [51].

2.2.2. Correlative dual-objective STORM and scanning EM (SEM) for unembedded samples

Next, we tested this imaging capability on the actin. The correlative 3D STORM and SEM could be the best choice for actin imaging because the embedding step required for TEM imaging of sectioned sample, has been known to cause the structural perturbations of cytoskeletal elements [52]. Although the small diameter and high packing density of actin filaments have limited the high-resolution light microscopy imaging of the individual actin filaments [13,53], recently developed dual-objective STORM could resolve individual actin filaments in cells with improved resolution of STORM [26,27]. By combining astigmatism imaging with a dual-objective scheme, <10-nm lateral resolution and <20-nm axial resolution can be obtained [26]. To image ultrastructure of actin filaments in cells, we combined dual-objective STORM with SEM (SE). To take full advantage of the high image resolution of dual-objective, we used normal glass coverslip (No.1.5) instead of photo-etched gridded coverslip (No.2). To easily find the same ROI with normal coverglass, we used the linear transformation of stage coordinates of the STORM setup and the EM setup. To generate the linear transformation,
we read the stage coordinates of four corners of a square coverglass in two setups (Figure 2.2.6C). Based on this transformation, we could easily calculate the stage coordinate of ROI in EM setup from that in STORM setup.
Figure 2.2.6. Correlative 3D dual-objective STORM and SEM. (A) Flowchart of the major steps in correlative 3D dual-objective STORM and SEM imaging of unembedded samples. (B) Schematic overview of the correlative 3D dual-objective STORM and SEM. (C) Scheme of coordinate conversion between STORM stage and EM stage to find the same region of interest (ROI).
For EM imaging, the actin filaments in cells have to be uncovered. The most commonly used way to remove the cell membrane is detergent lysis, and it was also used for actin filaments imaging by dual-objective STORM[26]. To combine these two imaging techniques, we optimized the dual-objective STORM protocol to the EM protocol[52] for actin filaments in cells simply by increasing the triton concentration(1%) in the first fixation-permeabilization step. COS-7 cells were washed in PBS twice, and fixed and permeabilized using 0.3% glutaraldehyde and 1% Triton X-100 in cytoskeleton buffer (CB: 10 mM MES pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM MgCl$_2$) for 5 min in the first step, followed by the second fixation step using 2% glutaraldehyde in CB for 15 min. After the extration and strong fixation, we stained actin filaments in the COS-7 cells with Alexa Fluor 647 dye-labeled phalloidin, which binds actin filaments with high specificity (Figure 2.2.6A,B). With this method, we could resolve the individual actin filaments in cells (Figure 2.2.8A,B).
Figure 2.2.7. 3D dual-objective STORM images of phalloidin-labeled actin in a BS-C-1 cell on a No. 1.5 glass coverslip. (A) 3D dual-objective STORM image of actin in a BS-C-1 cell on a No. 1.5 glass coverslip. (B) Magnified views of the boxed regions in (A). (C) Transverse profiles of localizations corresponding to regions boxed in red in (B). Blue bars: localization frequency measured from the STORM image. Red line: Gaussian fit of the blue bars.
Figure 2.2.8. Correlative 3D dual-objective STORM and SEM images of phalloidin-labeled actin in a BS-C-1 cell. (A) Correlative 3D dual-objective STORM and SEM images of phalloidin-labeled actin in a BS-C-1 cell. Left: STORM image. Right: Overlaid image. (B) Magnified views of the boxed regions in A.. Scale bars, 5 um(A) 1um (B).

By comparing the STORM image with EM image in magnified view, we could differentiate actin filaments from other cytoskeletal elements including microtubules and intermediate filaments, which were observed in EM image but not in STORM image. This comparison also allows us to differentiate actin bundles from microtubules, both of which look thick in EM image.
2.3. Correlative 3D STORM and transmission EM (TEM) for un-sectioned samples

2.3.1. The development of protocol for Correlative 3D STORM and transmission EM (TEM)

Next, we report correlative 3D STORM and TEM imaging for hydrated, unembedded samples. To image the same sample by both STORM and TEM, we used silicon nitride support films (SiN window), which are transparent to both visible light and electron beams (Figure 2.3.1).

Figure 2.3.1. Scheme of the SiN window and the sample mounting geometry.
In this method, similar to the correlative 3D STORM and SEM, neither STORM nor EM images were substantially compromised because STORM imaging was performed prior to any sample preparation required for EM imaging and most of sample preparations for either STORM or TEM were not changed from the standard protocols used for each imaging modality.

### 2.3.2. Applications of Correlative 3D STORM and TEM imaging

As the first target to test correlative 3D STORM and TEM, we imaged microtubules in BS-C-1 cells. For TEM imaging of the microtubules in cells, we used detergent lysis to remove the cell membrane, which was used in correlative dual-objective STORM and SEM imaging for actin filaments. We first performed 3D STORM imaging of the microtubules in cells plated on No. 1.5 coverslips to check for image quality without any concern of EM (Figure 2.3.2A-C). BS-C-1 cells were washed in PBS, and fixed and permeabilized using 0.3% glutaraldehyde and 0.25% Triton X-100 in cytoskeleton buffer (CB: 10 mM MES pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM MgCl$_2$) for 5 min in the first step, followed by the second fixation step using 2% glutaraldehyde in CB for 15 min. We then performed immuno-labeling of microtubules with tubulin antibodies and Alexa 405 and Alexa 647 co-labeled secondary antibodies. Individual microtubule filaments were clearly resolved by STORM (Figure 2.3.2 A,B). The hollow tubular shape of the immuno-stained microtubules was resolved in the transverse profile, which showed two peaks separated by 36 nm and agreed with the previous reported value[20] (Figure 2.3.2 C).
Figure 2.3.2. 3D STORM images of immunolabeled microtubules in a BS-C-1 cell. (A) 3D STORM image of microtubules in a BS-C-1 cell on a No. 1.5 glass coverslip. (B) Magnified views of the boxed regions in (A). (C) Transverse profiles of localizations corresponding to regions boxed in white in b. Blue bars: localization frequency measured from the STORM image. Red line: Gaussian fit of the blue bars.

For correlative 3D STORM and TEM, the sample was prepared in the same way, but on the SiN window. The SiN window was sandwiched between two No. 1.5 coverslips for imaging, as shown in Fig. 2.3.1. In this imaging geometry, because the cell sample is at a distance from the coverslip, we had to consider refractive index mismatch between the imaging buffer and the coverslip, which could cause substantial spherical aberration in STORM imaging[54]. To alleviate this effect, we used an index-matching imaging medium, which contains 60% sucrose and 5% glucose (refractive index ~1.45)[54]. The number of photons detected per switching cycle of the dye was reduced
by ~19% in this geometry compared to the case where the cell is directly plated on the No. 1.5 coverslip (Figure 2.3.3C). The 3D STORM image could still resolve hollow tubular shape of the microtubules, with 32 nm width (Figure 2.3.4A-C). After 3D STORM imaging, the sample was stained with 0.1% aqueous tannic acid and 0.2% uranyl acetate, dehydrated with graded ethanol steps, and coated with a Pt/Pd layer ~ 1nm thick, followed by TEM imaging. We correlated the 3D STORM image to the TEM image using SiN mesh in the SiN window as fiducial markers.
Figure 2.3.3. Correlative 3D STORM and TEM. (A) Flowchart of the major steps in correlative 3D STORM and TEM imaging of unembedded samples. (B) Schematic overview of the correlative STORM and TEM. (C) Normalized number of photons detected on the samples mounted on the normal No. 1.5 coverslip and the SiN window. The results are normalized to the average photon number obtained from samples on the No. 1.5 glass coverslip (photon number = 5233).
All of the microtubules observed in the 3D STORM image were also observed in the TEM image (Figure 2.3.4A,B). Other types of cytoskeletal elements that did not appear in the STORM image could also be identified in the TEM image, presumably corresponding to actin and intermediate filaments. In order to estimate how accurate the correlation is, we calculated the normalized cross-correlation between STORM and TEM images and displayed it as a surface plot (Figure 2.3.4D). A sharp peak in the correlation map is clearly observed at the center and a simple Gaussian fit to the profile yielded a displacement of 1.3 nm from the center and a FWHM of 44.5 nm.
Figure 2.3.4. Correlative 3D STORM and TEM images of immunolabeled microtubules in a BS-C-1 cell. (A) Correlative 3D STORM and TEM images of microtubules in a BS-C-1 cell. Left: STORM image. Right: TEM image. Middle: Overlaid image. (B) Magnified views of the boxed regions in (A). Red arrows in TEM image: thick filaments corresponding to microtubules. (C) Transverse profiles of localizations corresponding to regions boxed in white in (B). Blue bars: localization frequency measured from the STORM image. Red line: Gaussian fit of the blue bars. (D) Cross-correlation between STORM and TEM images. Inset in blue box: Magnified view of cross-correlation. Scale bars, 5 um in (A) and 500 nm in (B).
Next, we imaged mitochondria in cells with correlative 3D STORM and TEM (Figure 2.3.6.A,B). We first performed 3D STORM imaging of the mitochondria in cells plated on No. 1.5 coverslips to check for image quality without any concern of EM (Figure 2.3.5A-C). The cells were again plated on the SiN window and assembled in the imaging chamber as described above. We used the standard indirect immunological fluorescence method to stain the outer membrane of mitochondria in BS-C-1 cells, as described previously[54]. After fixation with 4% PFA, we stained Tom20, a outer mitochondrial membrane protein, using primary antibodies followed by Alexa 405 and Alexa 647 co-labeled secondary antibodies. When imaging the mitochondria in BSC-1 cells on the normal glass coverslip, the mitochondrial outer membrane appeared as a thin envelope that enclosed a hollow space in the xy cross-sections taken from the mid-plane of mitochondria (Figure 2.3.5C).

Figure 2.3.5. 3D STORM image of immunolabeled mitochondria in a BS-C-1 cell on a No. 1.5 glass coverslip.  (A) 3D STORM image of immunolabeled mitochondria in a BS-C-1 cell immuno-stained for TOM20 on a No. 1.5 glass coverslip. (B) Magnified views of the boxed regions in A. (C) xy cross-section of the 3D STORM image of mitochondria in the same region. Scale bars, 5 um in (A) and 500 nm in (B, C).
For correlative 3D STORM and TEM, the sample was prepared in the same way, but on the SiN window. We could observe the expected hollow shape of the mitochondria in the 3D STORM image and individual mitochondria correlated with their counterpart in the TEM image (Figure 2.3.6.A,B).

![Correlative 3D STORM and TEM images of immunolabeled mitochondria in a BS-C-1 cell](image)

**Figure 2.3.6. Correlative 3D STORM and TEM images of immunolabeled mitochondria in a BS-C-1 cell.** (A) Correlative 3D STORM and EM images of mitochondria in a BS-C-1 cell innumo-stained for TOM20. Left: STORM image. Right: Overlaid image. Part of SiN mesh is visible on the upper left corner of the image. (B) Magnified views of the boxed regions in A. (C) xy cross-section of the 3D STORM image of mitochondria in the same region. Scale bars, 5 um in (A) and 500 nm in (B).

The next target to test correlative 3D STORM and TEM was individual
filamentous virus as the small sized and fragile biological sample. For correlative 3D STORM and TEM, the virus sample was prepared in the same way as described previously, but on the SiN window. Although fluorescence brightness was decreased to 81% owing to the geometry of SiN window (Figure 2.3.1A-C, Figure 2.3.4C), the 3D STORM image could still resolve hollow shape of the virus filament (Figure 2.3.7B). After 3D STORM imaging, the sample was stained with 1% osmium tetroxide, dehydrated, coated by carbon and imaged by SEM(SE) microscopy. The overlay of the 3D STORM over the corresponding SEM(SE) images were highly correlated (Figure 2.3.7.A,C, right), suggesting that fixation, dehydration and carbon-coating steps, which were done after STORM imaging, did not cause structural rearrangement or significant volume change of the sample.
Figure 2.3.7. Correlative 3D STORM and TEM imaging of individual filamentous influenza viruses. (A,C) Correlative 3D STORM and SEM images of Udorn virus immuno-labeled for HA. Left: STORM image. Right: SEM image. Middle: Overlaid image. (B) Transverse profiles of localizations corresponding to regions in the white box in (A). Blue bars: localization frequency measured from the STORM image. Scale bars, 100 nm.
Figure 2.3.8. Correlative 3D STORM and TEM imaging of individual filamentous influenza viruses showing bulbous heads at the end. (A-C) Correlative 3D STORM and SEM images of Udorn virus immuno-labeled for HA. Left: STORM image. Right: SEM image. Middle: Overlaid image. Scale bars, 100 nm in (A,C) and 500 nm in (B).

Correlative images sometimes showed that the majority of filamentous viruses have bulbous heads at their leading ends, consistent with a previous cryo-tomography observation[55].
2.4. Correlative STORM and back scattered electron (BSE)-SEM imaging of embedded and sectioned samples

2.4.1. Developing the method for Correlative STORM and back scattered electron (BSE)-SEM imaging of embedded and sectioned samples

To probe the interior regions of thick biological samples using EM, the samples are typically embedded in resins and thin-sectioned using an ultramicrotome. To this end, we developed a correlative STORM and BSE-SEM imaging assay for resin-embedded and sectioned samples. We chose BSE-SEM as the EM imaging mode because it could be applied to samples mounted on glass coverslips, which is convenient for STORM imaging. It is also straightforward to extend the protocol describe here to correlative STORM and TEM imaging by using TEM grids instead of coverslips for mounting samples.

The major differences between this method and the methods describe earlier are two fold: 1) the embedding procedure and choice of embedding resins could affect the brightness and photoswitching properties of the fluorophores used for STORM imaging as well as the ultrastructural preservation, and 2) EM-related fixatives and stains need to be applied to the sample prior to embedding and sectioning, and hence could also affect the properties of the fluorophores. To minimize the effects of these factors on the STORM and EM image quality, we tested various types of resins, polymerization
strategies, EM fixatives and stains, and exploit a chemical etching approach to expose the epoxy-embedded dye to the imaging buffer to optimize photoswitching.

STORM imaging relies on the photoswitching and localization of individual fluorophores. For many dye molecules, such as Alexa 647, the switching performance can be enhanced by a primary thiol in the imaging buffer[19] (Scheme.1). Alexa 647 switched relatively poorly in resin embedded sections, likely because the plastic resin blocked the access of the thiol to the dye molecules. Our lab members, Hazen Babcock and Yari Sigal, therefore came up with an idea of an approach to expose the dyes in the embedded and sectioned sample to the imaging buffer by chemical etching the sections with sodium ethoxide, which has previously been used to enhance immuno-gold labeling[56]. This etching procedure substantially improved the switching performance of dye molecules(Figure 2.4.1). In the following examples, all fluorophore brightness measurements and STORM imaging of embedded and section samples were performed after etching.

\[
\text{Cy5} + \text{RS}^- \xrightleftharpoons{K_d} (\text{Cy5}\bullet\text{RS}^-) \xrightleftharpoons{\sigma I} (\text{Cy5}\bullet\text{RS}^-)^{EC} \xrightleftharpoons{k_f} \xrightarrow{k_0} \text{Dark State}
\]

**Scheme.1. Kinetic Pathway for Dark State Formation[19]**

**Figure 2.4.1. Schematic view of etching process on the resin.**
For embedding resins, we tested two epoxy-type resins, Ultrabed (Spurr-like) and Durcupan ACM as well as an acyclic-type resin, LR White. The acrylic resin is preferred for post-embedding immuno-labeling because the relatively porous structure of the acrylic resin partially preserves the antigenicity and allows antibodies greater penetration and access to epitopes[57]. On the other hand, the epoxy resins are generally considered better for ultrastructural preservation and for higher quality sectioning. Even though the acrylic resin partially preserves the antigenicity, the antibody labeling density was substantially lower than that obtained on unembedded samples and hence limited the resolution of the STORM images, which was also previously observed by our lab members, Hazen Babcock and Yari Sigal. We thus chose to immunolabel the samples prior to embedding in resins in this work. We measured the brightness (i.e. the number of photons per switching event) of the fluorescent dyes embedded in Ultrabed, Durcupan and LR White. Because the UV light-curing procedure typically used for curing LR White severely quenched the fluorescence, we used a chemical curing method instead of UV light. The measurement results (Figure 2.4.7A) indicate that the fluorescence signals of dyes are better preserved in the epoxy resins, Ultrabed and Durcupan, than in the acrylic resin LR white. Consequently, in the following experiments, we use Ultrabed as the embedding resin.

For resin-embedded samples, a strong fixative is essential to cross-link cellular structures and protect the tissue from major distortions that can be caused by the dehydration and resin embedding procedures. This is especially true for preserving membrane structures[58]. Many fixatives, such as osmium tetroxide and uranyl acetate both fix and stain. Others like tannic acid do not stain but instead act as a mordant to
enhance binding of metals to increase contrast in EM images. However, strong fixation conditions and some metals can quench fluorescence\cite{59}. For example, osmium tetroxide strongly quenches signals from fluorophores because it is a very strong oxidizing agent\cite{60} and it has been shown previously that signals from fluorescent proteins are reduced by more than 90% even with a very low concentration (0.1%) of osmium tetroxide\cite{39}. To obtain high-quality correlative STORM and EM images, we tested both an osmium free EM fixative/stain, containing a tannic acid (1%) - uranyl acetate (1%) combination and a non-oxidative osmium [osmium tetroxide (0.6%) and potassium ferricyanide (1.5%)] - uranyl acetate (1%) combination. Tannic acid was used in the first case in combination with uranyl acetate as uranyl acetate alone is not sufficient to generate high EM contrast of membranes\cite{46,58}. The concentrations were chosen such that the fluorescent signals of the dyes used here were not substantially quenched by the fixative/stain agents(Figure 2.4.2A,B).
Figure 2.4.2. Correlative STORM and SEM-BSE images of resin-embedded sections containing filamentous influenza viruses budding from infected cells. (A) Flowchart of the major steps in correlative 3D STORM and BSE-SEM imaging of embedded samples. (B) Schematic overview of the correlative STORM and SEM-BSE
As the acidic pH conditions typically accompanying these fixatives also quench the fluorophore signal, we thus adjusted pH of fixatives to maximize the fluorescence signal[60]. For example, when 1% aqueous tannic acid solution and 1% aqueous uranyl acetate solution (~pH 4) was used, the fluorescence intensity of the Alexa 647 dye was reduced by 35%, whereas, when the pH of the tannic acid and uranyl acetate solution was adjusted to 8.0 using Tris buffer, the fluorescence reduction was only 7%. Similar results were observed for the non-oxidative fixatives.

2.4.2. Correlative STORM and BSE-SEM imaging of filamentous influenza virus budding from cells.

As the first test of the correlative STORM and BSE-SEM method, we again imaged filamentous influenza virus budding from cells. We plated the cells on plastic coverslips as these coverslips can be relatively easily removed from the hardened resin block after polymerization. After virus inoculation, the infected cells were fixed by 4% PFA and 0.1% GA and immuno-labeled with anti-HA primary antibody and Alexa 647-labeled secondary antibody. The labeled sample was then post-fixed with 4% paraformaldehyde and 0.1% glutaraldehyde to covalently attach the immuno-label to the sample to minimize loss during dehydration and embedding. We used tannic acid – uranyl acetate to further fix and stain the sample so as to enhance the membrane contrast for EM imaging. We then dehydrated the fixed sample, embedded the sample in Ultrabed resin, and sectioned the sample at a thickness of ~70nm. Finally, we etched the sections with sodium ethoxide, dried the sections on the hot plate at 60 °C and rehydrated with
imaging buffer for STORM and then BSE-SEM imaging. The STORM and EM images are well correlated (Figure 2.4.3A,B and Figure 2.4.4A,B), and show the hollow tubular structures of viral filaments with expected width (Figure 2.4.3C and Figure 2.4.4C).

**Figure 2.4.3.** Correlative STORM and SEM-BSE images of resin-embedded sections containing an isolated filamentous influenza virus. (A) Correlative STORM and EM images of influenza infected A549 cells immuno-stained for HA. (B) Magnified views of the boxed regions in a (A). (C) Transverse profiles of localizations corresponding to regions in the white box in (B). Blue bars: localization frequency measured from the STORM image. Scale bars, 5 um in (A) and 500 nm in (B).
Figure 2.4.4. Correlative STORM and SEM-BSE images of resin-embedded sections containing filamentous influenza viruses budding from infected cells. (A) Correlative STORM and EM images of influenza infected A549 cells immuno-stained for HA. Left: STORM image. Right: SEM image. Middle: Overlaid image. (B) Magnified views of the boxed regions in a (A). Inset in yellow box: STORM image of an influenza virus filament, immuno-stained for HA, embedded in Ultrabed section without etching. (C) Magnified views of the boxed regions in (B). (D) Transverse profiles of localizations corresponding to regions in the white box in (C). Blue bars: localization frequency measured from the STORM image. Red line: Gaussian fit of the blue bars. (E) Cross-correlation between STORM and BSE-SEM images. Inset in blue box: Magnified view of cross-correlation. Scale bars, 5 um in (A) and 500 nm in (B, C).
The corresponding BSE-SEM image (Figure 2.4.3B and Figure 2.4.4A-C, right) also provides decent contrast at the membrane boundaries. The cross-correlation between the STORM and EM images (Figure 2.4.4.E) again appeared as a sharp peak close to the center, implying a high correlation between two images (displacement error of 2.5nm and FWHM of 45.9nm).

2.4.3. Correlative STORM and BSE-SEM imaging of an intracellular membrane-bound organelle

Next, we performed correlative STORM and BSE-SEM imaging of an intracellular membrane-bound organelle, the mitochondria. After fixation with 4% PFA and 0.1% GA, BS-C-1 cells were permeabilized with 0.2% Triton and immuno-stained for the mitochondria outer membrane protein Tom20 with primary antibodies and secondary antibodies doubly labeled with Alexa 405 and Alexa 647. The immuno-labeled cells were post-fixed with 4% PFA and 0.1% GA before further membrane fixation, staining, embedding and sectioning followed by STORM and then EM imaging (Figure 2.4.5).
Figure 2.4.5. Flowchart of the major steps in correlative 3D STORM and BSE-SEM imaging of embedded samples.
Figure 2.4.6. Correlative STORM and BSE-SEM images of immunolabeled mitochondria in a resin-embedded sections of a BS-C-1 cell. (A) Correlative 3D STORM and EM images of cells immuno-stained for TOM20. Left: STORM image. Right: SEM image. Middle: Overlaid image. Scale bars, 500 nm. (B) Magnified views of the boxed regions in a (A).
Because the EM contrast of intracellular membranes tends to be low after cell permeabilization, we used the stronger membrane fixative/stain, the non-oxidating osmium-uranyl acetate combination, here to enhance the membrane contrast of mitochondria. The correlated STORM and BSE-SEM images of mitochondria are shown in Figure 2.4.5-6, with the STORM images of TOM20 showing the expected outer membrane staining and the EM images show the typical cristae structure of mitochondria.

### 2.4.4. Photon number and number of switching cycles analysis

Since the localization precision of individual fluorophores and hence the STORM image resolution depend on the number of photons detected per switching cycle of the dye[20,23], we quantitatively characterized the effect of each sample preparation steps on the brightness of the fluorophores, i.e. the number of photons detected per switching event of Alexa 647 (Figure 2.4.7B,C).
Figure 2.4.7. The average photon numbers per switching event of dye molecules under various conditions used in the correlative STORM and EM imaging. (A) Normalized photon numbers in different resins. (B) Normalized photon numbers detected after various steps of sample treatment in the case of tannic acid - uranyl acetate fixed, Ultrabed-embedded samples. (C) Normalized photon numbers detected after various steps of sample treatment in the case of the osmium - potassium ferricyanide - uranyl acetate fixed, Ultrabed-embedded samples. The photon numbers in all cases are normalized to the control sample (photon number = 5233), which was post-fixed only with 4% PFA and 0.1% GA after immunolabeling without any additional membrane fixation (by osmium, tannic acid or uranyl acetate), dehydration, polymerization, sectioning or etching.
Figure 2.4.7B shows the fluorescence signal changes in the case where the tannic acid - uranyl acetate combination was used as the membrane fixative and stain. The tannic acid and uranyl acetate addition cause a 6% decrease in the photon number, and the following dehydration step induced another 8% decrease. Finally the resin embedding, sectioning and sodium ethoxide etching steps caused a further 18% reduction in the photon number, in which the resin embedding made the largest contribution (Figure 2.4.7B). Thus, photon number was overall reduced to 68% of the original value of Alexa 647 for the embedded and sectioned sample in this case. In the case where the osmium - potassium ferricyanide - uranyl acetate combination was used as the membrane fixative and stain, photon reduction was similar: 11% reduction by osmium - potassium ferricyanide - uranyl acetate, 5% by dehydration, and 20% by embedding, sectioning and etching. Overall, the fluorescence signal was reduced to 63% of the original value. We note that although a higher concentration of the membrane fixatives/stains would increase the EM contrast, it would lead to larger decrease in the brightness of the fluorophores. Interestingly, compared to photoactivatable fluorescent proteins, in which the fluorescence signal was reduced by more than 90% with only 0.1% of osmium tetroxide[39], photoswitchable dyes appeared to be much more robust when exposed to membrane fixatives/stains, leading to less of a compromise between STORM and EM imaging. We also quantitatively characterized the effect of each sample preparation steps on the number of switching cycles per fluorophore. In contrast to the photon number, the number of switching cycles did not substantially change after various steps of sample treatment from the control sample. In the control sample prior to any EM-related sample treatment, the Alexa 647 fluorophore switches 14.3 times on average before bleaching.
After the various EM-related sample preparation steps (treatment with EM fixatives/stains, dehydration, and embedding), the average numbers of switching cycles per fluorophore are 13.6-14.3.
2.5. Discussion

Here, we report several correlative STORM and EM assays for imaging both unembedded samples and resin-embedded, sectioned samples. For the unembedded samples, the best results were obtained by doing the EM-specific sample treatment, such as membrane fixation and negative staining, dehydration, and metal/carbon coating after STORM imaging. In this way, the conditions for STORM and EM imaging could be separately optimized. However, since multiple sample treatment steps occur between STORM and EM imaging, it is important to minimize the extraction or distortion of samples between these two imaging steps to produce highly correlated images. If the samples were not fixed sufficiently or if the dehydration procedure was too harsh or too long, the cellular ultrastructure could be degraded between STORM imaging and subsequent processing for EM imaging.

For the embedded samples, because EM-specific fixation and staining have to be applied to the sample prior to the resin-embedding step and STORM imaging is performed after embedding and sectioning, it is important to optimize the fixation/staining conditions and embedding resin materials such that the emission property of the fluorophores used for STORM imaging is not substantially altered, while still providing sufficient preservation and contrast to observe cellular ultrastructure in the EM image. Strong fixatives/stains could quench emission from fluorophores but weak fixatives/stains will not preserve ultrastructure or provide sufficient EM contrast. Compared to previous studies[39,43,46], we used stronger fixatives/stains to preserve
cellular ultrastructure and enhance membrane contrast in EM. We found that the fluorescence signal from the dyes were largely preserved under these strong fixation and staining conditions. Interestingly, organic dyes appeared to be much less perturbed by the fixatives than fluorescent proteins. The choices of embedding resins can also affect fluorescence emission and ultrastructure preservation. Instead of using acrylic resins [39,43,46], we used epoxy resins here because they are known to be better for ultrastructural preservation and sectioning. We also found the fluorescence signals of dye molecules to be better preserved in epoxy resins than in acrylic resins. Finally, because resin embedding can prevent the switching agents from reaching the embedded dyes and compromise the photoswitching property of the dyes, we also applied a chemical etching step which idea was initiated by our lab members, Hazen Babcock and Yari Sigal, to partially remove the resin material after sectioning and recover the photoswitching behavior of the dyes. Without this etching step, the switching of the dyes was substantially inhibited and the STORM image quality was substantially degraded. For example, when imaging filamentous influenza virus in embedded sections without etching, we found it difficult to switch off the Alexa 647 dye molecules in order to reach single-molecule imaging conditions. Often more than one dye molecule was on per diffraction-limited area, making it difficult to localize these molecules precisely. As a result, we were not able to resolve the hollow tubular shape of the viral envelope (Figure 2.4.4B, inset in the left panel).

We note that the assays reported here are complementary to, but not a replacement of, previously developed correlative super-resolution fluorescence and electron microscopy methods. For example, the use of a platinum replica could provide
substantially higher contrast for some cellular structures than negative stains[41,44,45,61,62], but can only be used to image structures near the surface of a specimen. For samples labeled with fluorescent proteins instead of dyes, embedding in epoxy resins largely quenches the fluorescence signal and acrylic resins need to be used[39]. Preservation of the signal of fluorescence proteins also requires weaker membrane fixatives/stains than what we used here[39]. Fluorescent dyes and proteins have distinct advantages and disadvantages in labeling biological samples – dyes are substantially brighter than fluorescent proteins and also allow endogenous proteins and nucleic acids to be labeled, but dye labeling is more difficult for living cells and also generates higher non-specific background than genetic fusion with fluorescent proteins. Genetic labeling with both fluorescent proteins and dyes (through a protein/peptide tag) allows correlative super-resolution fluorescence and cryo-EM imaging[47]. This correlative imaging mode has the advantage of avoiding chemical fixation and better preserving the native state of cellular structures, but the fluorescence image resolution is compromised due to the imaging geometry required for cryogenic samples. Future work is required to further explore the power of correlative imaging, for example, through the development of correlative live super-resolution fluorescence imaging and EM, either through fast fixation or freezing after fluorescence imaging or through liquid-chamber EM imaging[63,64]. We anticipate that correlative super-resolution fluorescence and electron microscopy will be a valuable tool for ultrastructural studies of many cellular processes.
2.6. Methods

2.6.1. Substrates cleaning

Various substrates were used depending on the experiments. For 3D STORM imaging without EM imaging, eight-well coverglass chambers (Labtek, 154534; Nunc) were used. Photo-etched gridded coverslips (Electron Microscopy Sciences, 72264-23) were used for correlative 3D STORM and SEM of hydrated, unembedded samples and the #1.5 square glass coverslip (Electron Microscopy Sciences, 72204-01) was used for correlative dual-objective STORM and SEM. For correlative 3D STORM and TEM of unembedded samples, silicon nitride support film (Ted Pella, 21515-10) was used. For correlative STORM and BSE-SEM imaging embedded, sectioned samples, plastic coverslips (Thermanox, 174950) were used for cell culture, which allow easy detachment of coverslips from the hardened resin block after polymerization, and #1.5 glass coverslips (Electron Microscopy Sciences, 72204-04) were used to mount the sections for STORM imaging. All of the substrates described above were cleaned by sonication for 20 min in 1 M aqueous potassium hydroxide and the washed thoroughly with MilliQ water, except for the silicon nitride support films, which were washed briefly with 1 M aqueous potassium hydroxide and MilliQ water without sonication because these thin films (40 - 200 nm thick) are fragile. The cleaned glass coverslips used for supporting the embedded sections were additionally glow-discharged using a Glow discharge system (Agar Scientific AGB8960) to make their surfaces hydrophilic. This helps to get the sections flat on the coverslips with fewer wrinkles. To reduce wrinkles of ultrathin sections, the
sections were often treated with chloroform.

### 2.6.2. Cell culture and virus infection

For cell culture, each coverslip was put in the 6-well, 12-well or 24-well flat-bottom cell culture plates (Corning costar) depending on the coverslip size. BS-C-1 cells (African Green monkey kidney epithelial cells, American Type Culture Collection (ATCC), CCL-26) were cultured in Eagle modified minimum essential medium (ATCC), supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (ATCC; penicillin and streptomycin). A549 cells (lung carcinoma cells, ATCC CCL-185) were cultured in high glucose Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (ATCC; penicillin and streptomycin). The cells were maintained in a humidified, 5% CO₂ environment at 37°C.

For virus infection, Udorn virus strain (a gift from Robert Lamb, Northwestern University, Evanston, IL) was used. A549 cells were infected with a MOI of 3 pfu/cell of A/Udorn/72 for 12 hrs at 37°C and fixed as described below.

### 2.6.3. Immunostaining and post-fixation

For imaging viruses budding from cells, Udorn-infected A549 cells were washed
in PBS (phosphate-buffered saline) twice after 12 hrs infection, and fixed with a mixture of 4% paraformaldehyde (Electron Microscopy Sciences, 15714) and 0.1% glutaraldehyde (Electron Microscopy Sciences, 16020) at room temperature for 15 minutes (for HA staining or HA and vRNP staining) or fixed with methanol at -20 °C for 2 min [65]. The cells were then washed with PBS and blocked with 3% (w/v) bovine serum albumin (Jackson ImmunoResearch Laboratories) in PBS for 30 min. For single-color HA staining, cells were incubated with goat HA primary antibodies (NR3118, BEI, Manassas, VA) in blocking buffer for 1 hr and Alexa 405 and Alexa 647 co-labeled bovine anti-goat secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 hr. For two color imaging, cells were stained with goat HA primary antibodies (or goat M1 primary antibodies (Abcam, ab20910) and mouse vRNP primary antibodies (Millipore, MAB8800) in blocking buffer for 1 hr, followed by Alexa 647 labeled bovine anti-goat secondary antibodies and Alexa 568 labeled anti-mouse secondary antibodies for 1 hr. The labeled cells were post-fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde at room temperature for 10 minutes and washed in PBS. For correlative STORM and BSE-SEM imaging of embedded samples, the post-fixed samples were further fixed and stained with 1% tannic acid (Electron Microscopy Sciences, 21700) in TRIS-maleate buffer (MB, Electron Microscopy Sciences, 11740) pH 6.0 for 10 min, washed with MB for 20 min, and then stained again with 1% uranyl acetate (Electron Microscopy Sciences, 22400) in MB, pH 6.0 for 10 min.

For mitochondria imaging, BS-C-1 cells were washed in PBS twice, fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde at room temperature for 15 min, and then reduced with 0.1% sodium borohydride for 7 min [59]. After washing with
PBS, the cells were permeabilized with 0.2% Triton X-100 and 3% (w/v) bovine serum albumin in PBS for 10 min, and then blocked with 3% (w/v) bovine serum albumin in PBS for 30 min. The cells were stained with rabbit anti-Tom20 (Santa Cruz Biotech, 2 μg/ml) in blocking buffer for 30 min, washed with PBS, and then stained with Alexa 405 and Alexa 647 labeled donkey anti-rabbit secondary antibodies (2 μg/ml) in blocking buffer for 1 hr. The labeled cells were then washed with PBS and post-fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for 10 min at room temperature. For correlative STORM and BSE-SEM imaging, the post-fixed samples were further fixed and stained with 0.6% osmium tetroxide aqueous solution (Electron Microscopy Sciences, 19152) for 7 min, reduced with 1.5% potassium ferricyanide for 10 min, washed with MB for 20 min, then stained again with 1% uranyl acetate in MB, pH 6.0 for 10 min.

For microtubule staining, BS-C-1 cells were washed in PBS twice, fixed and permeabilized using 0.3% glutaraldehyde and 0.25% Triton X-100 in cytoskeleton buffer (CB: 10 mM MES pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM MgCl₂) for 5 min in the first step, followed by a second fixation step using 2% glutaraldehyde in CB for 15 min. To reduce the background, the fixed cells were reduced by 0.1% NaBH₄ in PBS for 7 min[59]. Following washing with PBS for 1 hr, the cells were blocked with 3% (w/v) bovine serum albumin in PBS for 30 min. They were stained for 1 hr with rat anti-tubulin primary antibodies (ab6160; Abcam, clone YL1/2) in blocking buffer, washed with PBS, and then stained with Alexa 405 and Alexa 647 co-labeled donkey anti-rat secondary antibodies (2 μg/ml) in blocking buffer for 1 hr. The cells were then washed with PBS and post-fixed with 4% paraformaldehyde and 0.1%
glutaraldehyde in PBS for 10 min at room temperature.

2.6.4. Embedding, sectioning and etching

The fixed and stained cells were washed with double-distilled water three-times for 10 min and then dehydrated in a graded ethanol series (60%, 75%, and 90% ethanol for 5 min each, and followed by 100% ethanol twice for 10 min each). Following dehydration, the sample was infiltrated by progressive incubations with ethanol and UltraBed (a Spurr-like resin, Electron Microscopy Sciences, 14310) at ratios of 2:1, 1:1, and 1:2, followed by 100% resin. After 10 hr infiltration, the sample was embedded and polymerized in 100% UltraBed resin using the BEEM Embedding Capsules (Electron Microscopy Sciences, 69913-05) by baking in a 70 °C oven for 17 hrs. For Durcupan ACM embedding, the sample was infiltrated by progressive incubations with ethanol and Durcupan mixture (Electron Microscopy Sciences, 14040), at ratios of 2:1, 1:1, and 1:2, followed by 100% resin. After 10 hr infiltration, the sample was embedded and polymerized in 100% Durcupan resin by heating in a 70 °C oven for 17 hrs. For LR White embedding, the sample was infiltrated by progressive incubations with ethanol and LR White (Electron Microscopy Sciences, 14383) at ratios of 2:1, 1:1, and 1:2, followed by 100% resin. To preserve fluorescence, we titrated ethanolamine into LR White to neutralize the resin pH. We chose cold curing by chemical accelerator rather than UV polymerization or heat polymerization to minimize the fluorescence quenching. Following infiltration, the sample was polymerized at -20 °C overnight by adding a chemical accelerator (Electron Microscopy Sciences, 14385). The BEEM Embedding
Capsules and the plastic coverslip were removed from the hardened resin block by dipping them into liquid nitrogen. The plastic block was trimmed and sectioned with a Leica Ultracut ultramicrotome to obtain the ultrathin sections (70 nm thick). If the section has wrinkles or was not fully stretched, we exposed it to chloroform vapor to make fully stretched section without wrinkles. We placed the sections on a glow-discharged glass coverslips, followed by drying on a hot plate (60 °C) for 20 min. The dried sections were etched in 10% saturated sodium ethoxide for 30 sec, washed with double-distilled water and then dried again on a hot plate (60 °C) for 10 min.

2.6.5. STORM imaging

The samples were imaged in an imaging buffer containing 100 mM mercaptoethylamine(MEA) and an oxygen scavenger system [5% glucose (wt/vol), 0.5 mg/ml glucose oxidase (Sigma-Aldrich), and 40 mg/ml catalase (Sigma-Aldrich, C100-50MG)] in PBS at pH 8.5, which has a refractive index of 1.34. For correlative 3D STORM and TEM, higher refractive index (1.45) media was used, which contains 60% (wt/wt) sucrose and 5% (wt/wt) glucose in the imaging buffer described.

The samples were imaged with the 100 mM mercaptoethylamine(MEA) at pH 8.5, 5% glucose (wt/vol) and oxygen scavenging enzymes(0.5 mg/ml glucose oxidase (Sigma-Aldrich), and 40 mg/ml catalase (Sigma-Aldrich, C100-50MG) in PBS, which has a refractive index of 1.34. For correlative 3D STORM and TEM, higher refractive index (1.45) media was used, which contains 80% (vol/vol) glycerol and 60% (wt/wt)
sucrose solution and 5% (wt/wt) glucose with the same amount of mercaptoethylamine and oxygen scavenging enzymes as described above. To mount the SiN window sample, it was sandwiched between two glass coverslips by sealing with nail polish (Electron Microscopy Sciences, 72180). The photo-etched gridded coverslip for correlative 3D STORM and SEM, the glass coverslip for correlative dual-objective STORM and SEM, the ultrathin sections placed glass coverslip were mounted by sandwiching with another glass coverslip (No.1.5).

When SiN was used as the substrate for the sample, the SIN film was sandwiched between two glass coverslips. The SiN was first placed on a glass coverslip in an imaging buffer, assembled into a sample chamber with another No. 1.5 glass coverslip and the chamber was sealed by nail polish (Electron Microscopy Sciences, 72180) around the edges of a coverslip. When the photo-etched gridded coverslip was used as the substrate for the sample, it was also assembled into a sample chamber with a No. 1.5 glass coverslip with nail polish. The ultrathin sections of embedded samples were placed on one No. 1.5 glass coverslip, assembled into a sample chamber with another No. 1.5 glass coverslips and sealed the same way.

Single objective STORM experiments were performed on an Olympus IX71 inverted optical microscope using an Olympus UPlanSApo 100x, 1.4 NA oil immersion, as previously described (17). Alexa 647 dyes were excited using a 647 nm laser (MPB Comm Inc. 2RU-VFL-P-1500-647) and activated using a 405-nm laser (Cube 405-50C; Coherent). The emission from the A647 was filtered with a bandpass emission filter (Chroma, ET700/75) and was detected using an electron-multiplying charge-coupled
device (EMCCD) camera (Andor Technology, Ixon DU897) at a frame rate of 60 Hz. For 3D STORM imaging, a cylindrical lens with a focal length of 1 m (Thorlabs LJ1836L1-B or LJ1144L1-B) was inserted into the imaging optical path to create astigmatism for 3D imaging, as previously described (16). For two-color imaging of Alexa647 and Alexa568, 561nm and 657nm beams were used to excite A647 and A568 and they were activated by 405nm laser. The two emissions were separated by a 630-nm long-pass dichroic mounted on a commercial beamsplitting device (Dual-View; Photometrics), and filtered with two bandpass emission filters (FF01-607/70; Semrock and ET705/72m; Chroma) and was detected using an electron-multiplying charge-coupled device (EMCCD) camera (Andor Technology, Ixon DU897) at a frame rate of 60 Hz.

The dual-objective STORM setup has been previously described (23) (24). Briefly, the sample was mounted between two opposing objective lenses (Olympus Super Apochromat UPLSAPO 100x, oil immersion, NA 1.40) by combining the 2D translation stage with a 1D translation stage for 3D control of the sample position. Alexa 647 dyes were excited using the 647 nm line from Kr/Ar mixed gas laser (Innova 70C Spectrum, Coherent) and activated using the 405 nm solid state laser (CUBE 405-50C, Coherent). Fluorescence from Alexa647 collected by the two objectives were filtered separately with two 647-nm notch filters (Semrock NF01-543/647), and then imaged on two different areas of the same EMCCD camera (Andor iXon DU-897) at a frame rate of 60 Hz using two pairs of relay lenses. For 3D imaging, two cylindrical lenses(Thorlabs LJ1836L1-B or LJ1144L1-B) were inserted into each imaging optical paths. The two split movies from the two objectives were analyzed separately and after mapping the localizations from the second movie to the coordinates of the first movie, the final image was reconstructed by
determining each localization as a weighted averaged of the coordinates from each movie.

2.6.6. Electron microscopy imaging

After STORM imaging, the substrates with sample still attached were disassembled from the other coverslip by removing the nail polish with ethanol. The samples were washed with double-distilled water before subsequent EM sample preparation steps. All of the following EM sample preparations were performed between STORM and EM imaging.

For correlative dual-objective STORM and SEM(SE) imaging of actin and correlative 3D STORM and TEM imaging of microtubules, the sample was incubated in 0.1% aqueous tannic acid for 20 min at room temperature.

For correlative 3D STORM and TEM imaging of unembedded sample of microtubules, the sample was incubated in 0.1% aqueous tannic acid for 20 min at room temperature. After washing three times with distilled water for 10 min, it was incubated with 0.1-0.2% uranyl acetate in distilled water for 20 min at room temperature and washed again with distilled water for 10 min. The sample was then dehydrated in a graded ethanol series (60%, 75%, 90% and 100% ethanol for 5 min each, 0.2% uranyl acetate in 100% ethanol for 20 min, followed by 100% ethanol twice for 10 min each). The samples were further dried with hexamethyldisilazane for 15 min or by Critical Point Drying, and placed on a silicon wafer, followed by sputter-coating with 1 nm of
platinum/palladium (Sputter Coater, Cressington, 208HR). For TEM imaging, the samples were imaged in a TecnaiG2 Spirit BioTWIN at 80 kV with an AMT 2k CCD camera.

For correlative 3D STORM and TEM imaging of unembedded samples of mitochondria, the sample was post-fixed with 1% uranyl acetate (Electron Microscopy Sciences, 22400) in distilled water for 10 min at room temperature and washed with distilled water for 10 min. After dehydration in a graded ethanol series (60%, 75%, and 90% ethanol for 5 min each, followed by 100% ethanol twice for 10 min each), the samples were further dried with hexamethyldisilazane for 15 min or by Critical Point Drying. The samples were imaged in a TecnaiG2 Spirit BioTWIN at 80 kV with an AMT 2k CCD camera.

For correlative 3D STORM and SEM imaging of unembedded samples, the sample was further fixed in 1% osmium tetroxide aqueous solution (Electron Microscopy Sciences, 19152) for 10 min and then dehydrated in a graded ethanol series (60%, 75%, and 90% ethanol for 5 min each, and followed by 100% ethanol twice for 10 min each). The samples were further dried with Hexamethyldisilazane (HMDS, Electron Microscopy Sciences, 16782) for 15 min or by Critical Point Drying (CPD, Tousimis, Auto Samdri 815 Series A), and placed on a silicon wafer followed by carbon-coating with the carbon evaporator (HHV Auto306). The samples were imaged using an Ultra55 Field Emission Scanning Electron Microscope (FESEM, Zeiss) at 3 keV after identifying the same regions that were imaged with STORM by reading the number and grid on the photo-etched gridded coverslip.
For correlative STORM and BSE-SEM imaging of embedded samples, the sample sections were further stained by 2% uranyl acetate in ethanol solution for 10 min at room temperature, washed three times with distilled water, and then stained with Reynold’s lead citrate for 5 min at room temperature. The stained sections were rinsed again with distilled water and dried overnight. The coverslips with the sections were placed on a silicon wafer, and an additional carbon layer was sputtered on the sections for conductivity by using a carbon evaporator (HHV Auto306). The samples were imaged in a Zeiss Sigma Field Emission Scanning Electron Microscope (FESEM, Zeiss) with a the accelerating voltage of 7 keV by detecting back-scattered electrons and inverting black/white signal for easier visualization of the structure.

2.6.7. Image analysis

Before overlaying STORM and EM images, the brightness and contrast of the EM images was adjusted using PHOTOSHOP (Adobe Systems, San Jose, CA, U.S.A.) and the EM image was roughly rescaled until the scale bar matched the size of the scale bar on the STORM image. As a first rough alignment, the low magnification STORM image and the corresponding EM image were overlaid by rescaling, translation and rotating using PHOTOSHOP or the image registration function Control Point Registration in Matlab (The Math Works Inc., Natick, MA). Features visualized in both images could be used as fiduciary markers in this step, including gold fiduciary markers, the silicon nitride support mesh in the SiN window, dirt, the edge of the section and wrinkles in the section.
After the rough alignment, a refined alignment between two images was performed based on structures within the cells, such as virus filaments, mitochondria, and microtubules, by rescaling, translation and rotating the images using PHOTOSHOP and Matlab. In order to estimate how accurate the correlation is, we calculated the normalized cross-correlation between STORM and EM images. To compute the normalized cross-correlation between STORM and EM images, images were converted to grayscale images and the cross-correlation between the EM and STOMR images was calculated using the 'normxcorr2' function of Matlab and displayed as a surface plot.

2.6.8. Photon number and number of switching cycles analysis

To investigate the effect of sample preparation steps on the photon output of the dyes, we analyzed the average photon number associated with more than 2,000,000 detected molecular localizations (each corresponding to a switching event of the dye molecules in the field of view) for each condition. We also calculated the mean values from the single exponential fit of the photon number distributions and obtained similar results to the average values. The results were normalized against the control condition, in which sample was post-fixed only with 4% PFA and 0.1% GA after immunolabeling without any additional fixation (with osmium, tannic acid or uranyl acetate), dehydration, polymerization, sectioning or etching. In order to investigate the effect of sample preparation steps on the number of switching cycles per fluorophore, we analyzed more than 1,000 detected dye molecules for each condition. From the fluorescence time traces of individual dye molecules, switching events were identified and counted.

3.1. Introduction

In mammalian cells, purine levels are controlled by two coordinated mechanisms. The first mechanism, the salvage pathway maintains purine nucleotide levels under normal physiological conditions while the second mechanism, de novo biosynthetic pathways is upregulated during growth [66,67]. The observed multiple enzymes complex from the copurification experiment which is involved in the purine biosynthetic pathway has supported the possibility of presence of a protein complex responsible for protecting unstable intermediates to increase metabolic flux [68]. And recent studies provided direct evidences for the association of these proteins into a mesoscale assembly, called the purinosome [69,70]. This dynamic protein assembly structure forms reversibly in response to purine depletion and its formation increases de novo purine biosynthesis [69-71]. Interestingly, pyrimidine biosynthesis is also catalyzed by multi-functional protein suggesting an analogous phenotype [72].
Previous study of purinosome organization within the cell using conventional fluorescence microscopy showed that microtubule network plays a role in the assembly and/or stabilization of the purinosome while actin filaments appear not to be involved [73]. Since the interaction between purinosomes and microtubules were observed, our collaborator, Jarrod B. French in Steven J. Benkovic lab has speculated about the possible purinosome association with mitochondria as it is the microtubule-dependent cellular organelles. In order to investigate the precise localization and possible physical associations of purinosomes, our previous lab member, Sara Jones used 3D STORM because the diffraction-limited resolution of conventional fluorescence microscopy could mask some important details of the interactions between cellular structures [25,54]. The preliminary result obtained by our previous lab member, Sara Jones revealed a highly correlated spatial distribution of purinosomes and mitochondria (Figure 3.1) implying a direct physical association. Greater than 60% of purinosomes were located in close to mitochondria (Figure 3.1J) which was more than the expected value from random distribution of purinosomes throughout the cytoplasm. An association of purinosomes and mitochondria could be a synergistic relationship given the fact that purines are needed to generate ATP in the mitochondria and that ATP is required in the de novo purine biosynthesis. In addition, one-carbon units generated by the mitochondrial conversion of serine to formate can be incorporated into the purine ring during de novo biosynthesis. These potential functional synergies between purinosomes and mitochondria would be extremely beneficial to the cell.
Figure 3.1. Super-resolution imaging of purinosomes and mitochondria. (A) 2D projection of a 3D STORM image showing purinosomes (mEos2-FGAMS, magenta) and mitochondria (anti-Tom20, green) in a HeLa cell. (B) Zoom in of the boxed region in panel a showing the close interaction between the two structures. (C) An xy-cross section of the region in b demonstrates that purinosomes and mitochondria are in the same axial plane within the cell. Note the hollowness of the mitochondria, corresponding to the outer membrane stain. (D) Zoomed image of the upper boxed region in B, with xz-cross sections along the dotted lines shown in the panels below (E, F). The purinosomes sit nestled within the curve of the mitochondria or slightly wrapping around it. A conventional fluorescence image (G) and corresponding 2D projection STORM image (H) of the lower boxed region in b showing the improved resolution. Accordingly, the purinoosome can again be seen to sit between neighboring mitochondria in the xz-cross section (I). (J) Quantification of association between mEos2-FGAMS and mitochondria. The calculated percentage (STORM) was compared to a randomized purinosome distribution within the cell (Random) (N=26 cells, p<<0.0001). Scale bars, 5um (A), 1um (B-C), 250 nm(D-I).
In order to investigate the functional association of purinosomes with mitochondria, our collaborator, Huanyun Deng and Haibei Hu in Ye Fang Lab, examined the possible kinases which may play a role in purinosome formation using a two-step dynamic mass redistribution (DMR) assay[74] and one of the identified kinases from this screening is master regulators of cellular metabolism, the mechanistic target of rapamycin (mTOR). mTOR is involved in regulating many cellular processes, including nucleotide metabolism and it has been demonstrated that mTOR actively associate with mitochondria-associated ER membranes and modulate mitochondrial physiology[72,75-77]. Therefore, the previous data suggest the role of mTOR in modulating purine biosynthesis. Although several evidences support the mTOR-mediated functional association of purinosomes and mitochondria, further investigation to directly examine the effect of mTOR inhibition on the association of purinosomes and mitochondria is required to confirm the role of mTOR in purinosome formation.
3.2. Result

3.2.1. Imaging of endogeneous purinosomes using LND fibroblast and mMaple3

The purinosomes can be a valuable tool to study the disorders of purine metabolism, particularly related to the purine synthesis mechanism. Several disorders of purine metabolism have been reported with variable clinical symptoms [78,79]. Although it is important to understand the pathogenesis of these disorders for developing therapeutic approaches, less information is available, especially about their metabolic relationships. Among them, Lesch-Nyhan disease is the neurogenetic disorder caused by a wide variety of mutations in the HPRT1 gene which encodes hypoxanthine-guanine phosphoribosyl transferase (HGPRT), an enzyme involved in purine metabolism [80-84]. As a result, Lesch-Nyhan disease has been used as a model of genotype–phenotype relations, expanding our understanding of genotype–phenotype correlations in many other neurogenetic disorders [82]. The cell based study of LND disease can be advantageous to explore specific aspects of metabolism responsible for several consequent changes, such as the failure of purine recycling, an accumulation of purine waste products and accelerated synthesis of purines [85], because the experiment environment can be tightly controlled.

Recent studies also reported possible problems in the previous experiments using purine-depleted medium to exhibit purinosome formation in HeLa cells, which condition may have induced the aggregation of the recombinant proteins resulting in cellular stress [86,87]. Although previous studies showed that the increase in number of purinosome-positive cells in HeLa cells is not the stress granules as the result of extra stress on the cells [88], the need of observation of endogenous purinosomes without the purine-
depleted condition has been increased. For these two reasons, we studied a different cell line, HGPRT-deficient fibroblast, which exhibit a LND phenotype, relies primarily on the de novo purine biosynthetic pathway to generate purine nucleotides [80-84]. Because LND fibroblast doesn't require the purine-depleted condition to exhibit the purinosome formation, it could be an ideal model system to study purinosomes. In order to probe the endogenous purinosomes normally expressed in LND cells without purine-depleted condition, we performed STORM imaging by expression of mEos2 tagged FGAMS in LND cells. mEos2 tagged FGAMS in LND cells were expressed by our collaborators, Chung Yu Chan in Steven J. Benkovic lab. We obtained the STORM images of purinosomes and they revealed homogeneously distributed purinosomes formation inside the cells similar to the purinosomes in HeLa cells. We investigated the average size of purinosomes from the STORM images collected using HeLa cells and LND fibroblast. STORM images collected using HeLa cells revealed the mean diameter value of 570 nm and the mean number of 110, and those using LND fibroblast revealed the mean diameter value of 575 nm and the mean number of 109(Figure 3.2A-B). Because similar purinosome diameter and number between two different cell lines were noted, we found that the observed endogenous purinosomes without the purine-depleted conditions show the similar physical features of purinosomes induced by purine-depleted conditions in HeLa cells and hence the observed purinosomes from HeLa cells are not the stress granules as the result of extra stress on the cells. This observation is also consistent with previous biochemical results of the accelerated rate of de novo purine biosynthesis in LND cells [89], suggesting enhanced endogenous purine synthesis resulted from a single gene defect.
Figure 3.2. Distribution of purinosome size and number using different fluorophore and different cell lines. (A) Distribution of purinosome size using different cell lines. Images collected using HeLa cells (blue bars) were compared to images collected using LND cells (red bars). (B) Distribution of purinosome number using different cell lines. Images collected using HeLa cells (blue bars) were compared to images collected using LND cells (red bars). No observable difference in purinosome diameter or number was noted. (C) Distribution of purinosome size using different fluorophore. Images collected using FGAMS-mEos2 at 560 nm (blue bars) were compared to images collected using FGAMS-mMaple3 at 565 nm (red bars). (D) Distribution of purinosome number using different fluorophore. Images collected using FGAMS-mEos2 at 560 nm (blue bars) were compared to images collected using FGAMS-mMaple3 at 565 nm (red bars).

In the previous imaging experiments, purinosomes could be visualized at high resolution using FGAMS linked to the photoactivable fluorescent protein mEos2 (FGAMS-mEos2). However, some problems with aggregation have been reported at high
densities. Although mEos2 has been developed as monomeric fluorescent proteins, some residual tendency to dimerize was reported, which could potentially lead to undesired aggregation of cellular proteins [90]. We thus also tried alternative photoactivatable fluorescent protein, mMaple3, which were found to have lower dimerization affinity [90]. In order to perform STORM imaging experiments, our collaborators, Chung Yu Chan in Steven J. Benkovic lab, have constructed vectors that enable the expression of mMaple3 -tagged versions of FGAMS. We investigate the average size and number of purinosomes per cell from the STORM images collected using FGAMS-mEos2 and FGAMS-mMaple3 in LND cells. STORM images collected using FGAMS-mEos2 revealed the mean diameter value of 560 nm and the mean number of 96. STORM images collected using FGAMS-mMaple3 revealed the mean diameter value of 565 nm and the mean number of 113 (Figure 3.2C-D). No observable difference in purinosome diameter or number was noted. Therefore, we found that fluorophore identity did not affect the physical features of the observed purinosomes.

Next, we studied the spatial relationship between pruinosomes and mitochondria in LND cells. Purinosomes were imaged via expression of mMaple3 tagged FGAMS using LND cells in order to remove the possible effect of protein aggregations. Two color STORM images were then collected of cells exhibiting purinosomes and immunostained for the TOM20 using Alexa 647 dyes. The STORM image clearly resolved mitochondria and purinosomes, allowing a precise determination of their spatial relation (Figure 3.3A-C). The purinosomes often contacted mitochondria, consistent with highly correlated spatial distribution of purinosomes and mitochondria which were previously observed with the STORM images via expression of mEos2 tagged FGAMS using HeLa cells. We
again measured the degree of the colocalization between two structures and compared it with the value from the image of randomly distributed purinosomes. We found that greater than 60% of purinosomes were located in close, sub diffraction spatial proximity (<100 nm) to mitochondria and this value is more than the expected value from the image of randomly distributed purinosomes (Figure 3.3D). This result is consistent with the result value from the STORM images of mEos2 tagged FGAMS in HeLa cells, confirming the physical association between endogeneous purinosomes and mitochondria.

**Figure 3.3. Super-resolution imaging of purinosomes and mitochondria.** (A) 2D STORM image showing purinosomes (mMaple3-FGAMS, green) and mitochondria (anti-Tom20, red) in a LND cell. (B,C) Zoom in of the boxed region in panel a showing the close interaction between the two structures. (D) Quantification of association between mMaple3-FGAMS and mitochondria. The calculated percentage (STORM) was compared to a randomized purinosome distribution within the cell (Random) (N=43 cells, p<<0.0001). Scale bars, 5um (A), 1um (B-C).

### 3.2.2. Spatial relationship between purinosomes and microtubules

Some of the recent studies of the purinosome have suggested that purinosomes are associated with portions of the microtubule network [73,91]. However, a spatial relationship could be obscured in the conventional fluorescence images especially when
microtubules are densely packed [73]. In order to investigate the physical association between purinosomes and microtubules more precisely, we generated super-resolution images of purinosomes and microtubules using dual-color STORM which allows a more precise determination of their spatial relation as compared to the conventional fluorescence image. To map their spatial relation, we performed two-color STORM by staining tubulin and expression of mMaple3 tagged FGAMS in LND cells in order to remove the possible effect of protein aggregations. mMaple3 tagged FGAMS in LND cells were expressed by our collaborators, Chung Yu Chan in Steven J. Benkovic lab. We obtained the two color STORM images of purinosomes and microtubules and they revealed a highly correlated spatial distribution of endogenous purinosomes and microtubules such that greater than 80% of purinosomes were located in close, subdiffraction spatial proximity (<100 nm) to microtubules (Figure 3.4A-C). This is still more than the expected value from the image of randomly distributed purinosomes throughout the cytoplasm although the expected value from the images of randomly distributed purinosomes is relatively high due to the high density of microtubules inside a cell (Figure 3.4D). This high colocalization value implies significant interaction between two structures and suggests that the purinosomes may be trafficked throughout the cells on microtubule tracks. This type of intracellular trafficking is known to be facilitated by motor proteins such as kinesins and dyneins [92]. Previous studies using pulldown experiments reported that cytoplasmic dynein and dynactin were associated with FGAMS only under conditions that promote purinosome formation supporting this significant interaction between two structures [69,93].
Figure 3.4. Super-resolution imaging of purinosomes and microtubules. (A) 2D STORM image showing purinosomes (mMaple3-FGAMS, green) and microtubules (anti-tubulin, red) in a LND cell. (B,C) Zoom in of the boxed region in panel a showing the close interaction between the two structures. (D) Quantification of association between mMaple3-FGAMS and microtubules. The calculated percentage (STORM) was compared to a randomized purinosome distribution within the cell (Random) (N=20 cells, p<<0.0001). Scale bars, 5um (A), 1um (B-C).
3.2.3. Functional characteristics of the association between purinosomes and mitochondria

To further investigate the physical association of purinosomes with mitochondria we examined the possible mediator between mitochondria and purinosomes, mTOR. To confirm that the extent of physical association between these structures correlated with mTOR activity, two color 3D STORM images of purinosomes and mitochondria were imaged in the presence of mTOR inhibitor, rapamycin. Our collaborators, Chung Yu Chan in Steven J. Benkovic lab, have constructed vectors that enable the expression of mEos2-tagged versions of FGAMS and the proteins were expressed in HeLa cells. We collected the two-color STORM images for that sample and analyzed the degree of colocalization. Colocalization analysis revealed that fractional colocalization between purinosomes and mitochondria decreased with increasing concentration of rapamycin (Figure 3.5A), while purinosome size and number was unchanged up to concentrations of 500 nM (Figure 3.5B-C). This data suggests the function of mTOR in regulating purine biosynthesis by controlling the recruitment of purinosomes to mitochondria.
Figure 3.5. Relationship between purinosomes and mitochondria is mediated by mTOR. (A) The percentage of co-localized purinosomes and mitochondria (solid diamonds) decreases with increasing rapamycin concentrations as compared to a random distribution (solid circles). (B-C) Change in purinosome size and diameter with rapamycin treatment. Purinosome mean diameter and mean number in cells remained constant for rapamycin treatments up to 500 nM.

Similar mTOR-mediated stimulation of nucleotide metabolism were observed in pyrimidine synthesis, including the mechanism of control exerted by mTOR on pyrimidine metabolism and the mTOR-stimulated oligomerization of the enzyme CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase) which is involved in the de novo pyrimidine synthesis [72,75]. A further evidence for the relationship between nucleotide metabolism and the mitochondria is a mitochondrial
enzyme, dihydroorotate dehydrogenase, which is also involved in the pyrimidine biosynthesis. Therefore, the results reported here support the potential role of mTOR in the stimulation of nucleotides synthesis via a mechanism which is involved in the control of protein association and localization.
3.3. Discussions

We first demonstrate super-resolution imaging of endogenous purinosomes with other cellular components in HGPRT-deficient LND fibroblasts. STORM images of purinosomes and following cluster analysis revealed the similar purinosome formation in LND cells in terms of size and number with the purinosomes induced in HeLa cells under purine-depleted condition. This observation confirms that they were not the stress granules, suggesting its potential application as an ideal system to study the endogenous purinosome. This also can be a strong and direct evidence for the accelerated rate of *de novo* purine biosynthesis in HGPRT-deficient LND fibroblasts, which has been suggested from the biochemical results [89]. Next, we demonstrated highly correlated spatial distribution of purinosomes and mitochondria, which is consistent with the previous HeLa cells case, confirming the physical association between endogenous purinosomes and mitochondria.

We also investigated the endogenous purinosome association with microtubules using LND cells. The co-localization of purinosomes with microtubules suggests that microtubules play a role in purinosome trafficking. The precise role of the microtubules, however, remains unclear, for example, whether assembled purinosomes or purinosome intermediates are trafficked along the microtubules throughout the cell and how the dynamics of assembly occur on the microtubules. One possible mechanism of purinosome assembly and dynamics involves purinosomes forming at random locations inside the cell and stabilized once bound to microtubules, and are then trafficked along the microtubules in order to meet the cellular metabolic needs. A second possibility is that purinosomes are formed with the aid of microtubules in the assembly of individual
purinosome components, may be in close association with the mitochondria which is directly at their point of need. These possibilities can be tested by examining purinosome formation and trafficking using live-cell conventional and super-resolution imaging.

We anticipate that LND cells can be further used for the endogenous purinosome study, for example, molecular basis for the interaction between purinosome and other cellular components by identifying the spatially intermediate enzymes within a purinosome between two structures, which could be potentially used as the target for innovative therapeutic approaches. The cell based imaging study of LND disease can be also useful for the comparative studies of the different neurological symptoms displayed by persons afflicted with the Lesch-Nyhan disease related to the purine metabolism.

The control of nucleotide synthesis and management of the nucleotide level in a dynamic microenvironment are important cellular processes for cellular activity and metabolism. They can be regulated by post-translational control over metabolic flux. Finally, this study demonstrates one such mechanism of control. The relationship between mitochondrial functional and nucleotide metabolism which is essential for cell growth has been also suggested from the previous studies of mitochondria-related pyrimidine biosynthesis. The preliminary results from our previous lab member, Sara Jones and our collaborator, Jarrod B. French in Steven J. Benkovic Lab also support this hypothesis. For example, the co-localization of purinosomes with mitochondria suggests a functional synergy relationship between mitochondrial functional and purinosomes and the effect of mTOR inhibition on the purinosomes implies mTOR-mediated functional association between purinosomes and mitochondria. In this work, we could confirm the addressed idea of mTOR-mediated functional association of purinosomes and mitochondria. The
decreased colocalization between purinosomes and mitochondria by the mTOR inhibitor treatment clearly suggest that mTOR signaling pathway plays a primary role in the stimulation of metabolic flux of nucleotides by the control of protein association and localization. Such regulation can increase the metabolic efficiency, protect unstable intermediate and minimize the off-target effects by the spatial and temporal control of enzymes and enzyme complexes.
3.4. Methods

3.4.1. Constructs used for imaging experiments.

FGAMS-mEos2 and FGAMS-mMaple3 were constructed from FGAMS-GFP using standard molecular biology techniques.

3.4.2. Cell Culture

HeLa cells (ATCC) were maintained in ‘purine depleted’ conditions (RPMI 1640 with L-glutamine supplemented with 5% dialyzed FBS)[69]. The cells were transfected in OPTI-MEM using Lipofectamine 2000 (Life Technologies) following the manufacturer’s protocol. LND fibroblasts were grown in normal media and electroporated using the Neon Transfection SystemMPK5000 (Life Technologies) following the manufacturer’s optimized protocol. Reactions were performed under the conditions of 1400 V/20 ms/1 pulse. Cells were then transferred to Minimum Essential Medium (Corning) supplemented with 10% (vol/vol) FBS (Atlanta Biological). The cells were fixed 20 – 30 hours post transfection and imaged after that.

3.4.3. Cell fixation and immunostaining

Cells were grown as detailed above and washed 3 times with phosphate buffered saline (PBS). They were fixed with freshly made 3% paraformaldehyde (Electron Microscopy Sciences) and 0.1% glutaraldehyde (Electron Microscopy sciences) in PBS for 15 – 20 minutes at room temperature. Cells were then rinsed twice with PBS
and reduced with a solution of 0.1% Sodium Borohydride for 5 minutes and rinsed an additional three times with PBS. After washing, the samples were blocked and permeabilized with 3% Bovine Serum Albumin (IgG-free, Jackson ImmunoResearch) and 0.2% Triton X-100 in PBS for 60 minutes. They were incubated for 60 minutes with primary and secondary antibodies which were diluted in the same blocking buffer at room temperature. Cells were washed three times with a wash solution (0.2% BSA and 0.05% TritonX-100 in PBS), 5 minutes per wash between antibody incubations. After incubation cells were again washed and then post-fixed the labeled dyes with 3% paraformaldehyde and 0.1% glutaraldehyde for 10 minutes. After post-fixation, samples were rinsed three times in PBS and stored at 4°C or imaged immediately. Antibodies used include: anti-Tom20 (Santa Cruz Biotechnologies, sc-11415 or sc-17764) and rat anti-tubulin primary antibodies (ab6160; Abcam, clone YL1/2).

### 3.4.4. STORM imaging and analysis

The samples were imaged in an imaging buffer containing 100 mM mercaptoethylamine (MEA) and an oxygen scavenger system [5% glucose (wt/vol), 0.5 mg/ml glucose oxidase (Sigma-Aldrich), and 40 mg/ml catalase (Sigma-Aldrich, C100-50MG)] in PBS at pH 8.5, which has a refractive index of 1.34. STORM experiments were performed on an Olympus IX71 inverted optical microscope fitted with an Olympus UPlanSApo 100x, 1.4 NA oil immersion, as previously described[25,54]. Alexa 647 dyes were excited using a 647 nm laser (MPB Comm Inc. 2RU-VFL-P-1500-647) and activated using a 405-nm laser (Cube 405-50C; Coherent). The emission from the Alexa 647 was filtered with a bandpass emission filter (Chroma, ET700/75) and was detected using an electron-multiplying charge-
coupled device (EMCCD) camera (Andor Technology, Ixon DU897) at a frame rate of 60 Hz. For two-color imaging of Alexa 647 and mEos2 or mMaple3, 657nm and 561nm beams were used to excite Alexa 647 and mEos2 (or mMaple3), respectively, and the dyes and fluorescent proteins were activated by 405nm laser. The emissions from the two dyes were separated by a 630-nm long-pass dichroic mounted on a commercial beamsplitting device (Dual-View; Photometrics), filtered with two bandpass emission filters (FF01-607/70; Semrock and ET705/72m; Chroma) and detected on two different regions of the EMCCD camera at a frame rate of 60 Hz. For registration of two-color images of Alexa647 and mEos2 (or mMaple3), the two STORM images were aligned by a 3rd order polynomial warping map in 3D obtained from calibration images of 100 nm Tetraspeck fluorescent beads.

Colocalization between purinosomes and mitochondria was analyzed using custom written Matlab code. High-resolution images (~25.6 nm per pixel) of 2D projections of 3D STORM images of both mitochondria and purinosomes were median-filtered and subjected to intensity thresholding by Otsu’s thresholding algorithm. Object boundaries were dilated by 1 pixel using 8-point connectivity to account for rounding errors in boundary identification. The Euclidean distance between mitochondria-containing pixels and purinosome-containing pixels was determined and then it was used to determine the overlap matrix. The positive colocalization was defined as the mitochondria-purinosome pixel distances of less than 100nm. Considering the uncertainty in positional location (spatial resolution) and some spatial separation from the marker proteins, this number was chosen. In order to check how meaningful this colocalization number is, we also determined the expected value for a randomized purinosome distribution. To randomize the purinosome distribution, the cell and nuclear boundary
were defined and the centroid position of each identified purinosome in the above analysis was randomly assigned to a pixel location within defined cytoplasmic area while leaving all other shape and size parameters constant. The purinosome distribution in each individual original image was randomized and the mean value of colocalization from the ten randomized distribution images were calculated to compare with the true mitochondria distribution of the original image.
References


