CELLULAR RESPONSES TO EXTERNAL THREATS PROBED BY SUPER-RESOLUTION MICROSCOPY

BY

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DISSERTATION

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Abstract

Fluorescence microscopy has been an essential tool in biology. However, its imaging resolution has been limited around >200 nm in lateral dimensions, and >500 nm in axial dimension, leaving many biological structures too small to study. However the recent developments of several super-resolution techniques, this limit has been overcome. Here we present our approach to this matter, using our three-dimensional (3D) multicolor super-resolved single fluorophore microscopy. Especially we report imaging of a near-IR dye for imaging thick and dense structures for multicolor colocalization studies. Then we present three applications of the super-resolution imaging technique to the following three biological systems.

Viral infection in mammalian cells triggers the immune response, a cascade of antiviral signaling proteins. For double stranded RNA (dsRNA) virus, the innate immune response starts from retinoic acid-inducible gene-I (RIG-I) protein which is a dsRNA sensor. For certain types of viruses, RIG-I localizes in cytoplasmic granular aggregates called as antiviral granules (AVGs). We infected/transfected cells by influenza virus lacking NS1 (IAVΔNS1) or polyinosinic:polycytidylic acid (poly I:C) to induce AVGs. We found in AVGs, RIG-I forms clusters of around 110nm in diameter. Then we treated intact cells with various stress conditions to from stress granules (SGs) and could also observe RIG-I clusters in SGs. RIG-I was also clustered in intact cells, but the clustering percentage and diameter were small than in AVGs or SGs, so we conclude that the intrinsically clustered RIG-I relocalizes into granular structures upon external stimuli, and its clustering is enhanced. To verify RIG-I clustering, we imaged TIAR, a marker for SGs, which showed much less clustering. Also we conducted various tests on our clustering algorithm, as well as structural illumination microscopy (SIM) imaging that all support the idea of the clustering of RIG-I.

In bacterial cells, upon vast global DNA damage, an error-prone DNA repair response called SOS response occurs. SOS response is initiated by RecA, a protein essential for maintenance of DNA. It was reported that RecA forms a bundle to connect uncut and cut locus of in the event of double strand break, supporting the idea that RecA mediates homology search. We report that
RecA also forms bundles in the SOS response, and these bundles are ribbon-like structures, i.e. flat at one side and wide at the other side, hypothetically wrapping around DNA damaged sites.

The conventional approach of applying broad-spectrum antibiotics to treat bacterial infections contributes to the emerging of antibiotic resistance. To cope with this, species specific and narrow-spectrum antibiotics draw attention. Plantazolicin (PZN) is a natural antibiotic that is highly specific against B. anthracis which is the agent of anthrax and a category A priority pathogen, but the mechanism of how PZN kills B. anthracis has been unknown. Recent investigation showed PZN depolarizes B. anthracis membrane, and it was supported by the super-resolution imaging that PZN foci localize on the membrane of cells.
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Chapter 1
Super-resolved single-fluorophore microscopy

Introduction

Fluorescence microscopy has been an essential tool in biology due to its several advantages over other imaging techniques. Live cell imaging is possible due to the noninvasive nature of light, in contrast to electron microscopy where specially treated fixed cells are available for imaging. Also target specificity by techniques such as immunofluorescence, fluorescence in situ hybridization (FISH), and genetically encoded fluorescent tags against certain proteins or nucleotides makes it possible to study localizations of targets in individual cells. In contrast, electron microscopy has been mostly limited in image specific targets, and bulk measurements such as quantitative polymerase chain reaction (qPCR) or flow cytometry. Furthermore, fluorescent microscopy is capable of imaging multiple targets by utilizing different wavelengths of light to separate the signals into different channels. This enables studying the interactions of multiple targets [1].

In spite of many advantages, the imaging resolution of conventional fluorescence microscopy has been limited around >200 nm in lateral dimensions, and >500 nm in axial dimension. This made researchers unable to study effectively many biological structures in nm range in size and interactions [2]. However the recent developments of several super-resolution techniques provided different ways to overcome this limitation. They include super-resolved ensemble fluorophore microscopy such as Stimulated Emission Depletion (STED) microscopy [3], Structural Illumination Microscopy (SIM) [4], or super-resolved single-fluorophore microscopy
such as Photoactivated Localization Microscopy (PALM) [5, 6], Stochastic Optical Reconstruction Microscopy (STORM) [7] or Point Accumulation for Imaging in Nanoscale Topography (PAINT) [8]. In this report, we present our approach to this matter, using our super-resolved single fluorophore microscopy.

**The resolution limit of optical microscopy [9]**

**Abbe’s theory**

To demonstrate the effect of diffraction, let’s assume a diffraction grating as an object (Fig. 1A). The set of plane waves from the diffraction grating are refracted by a lens so that they converge individually as $S_i$s in the focal plane $F$. They continue and form the image in the imaging plane $I$. The image is composed of interference pattern (Fig. 1B).

The process of image formation can be seen as two stages. In the first stage we can consider $S_i$s as an imaginary diffraction pattern in the plane $F$. Then the image on the plane $I$ can be viewed as a diffraction pattern coming from $S_i$s, the equally spaced point sources. Each pair of $S_j$ and $S_j$ produces Young’s fringes in the plane $I$. Then

$$\theta_j \approx \sin \theta_j = j\lambda/d$$

, where $\lambda$ is the wavelength of the light and $d$ is the spacing of the grating, and here small angle-approximation is used. Then also

$$\theta_j \approx \tan \theta_j = h_j/U$$

$$\theta'_j \approx \tan \theta'_j = h_j/V$$

, where $h_j$ is the distance from the center of the lens to the light path, $U$ is the distance between the object and the lens, $V$ is the distance from the lens to the image plane $I$, and $\theta'_j$ is the convergence angle of the light from $S_j$ to the plane $I$. Then

$$\theta'_j \approx U\theta_j/V$$

The waves from $S_1$ and $S_1$ converge on $I$ and form periodic fringes with spacing
\[ d'_1 = \frac{\lambda}{\sin \theta'_1} \approx \frac{\lambda V}{\theta_j U} = \left( \frac{V}{U} \right) d \]

The first order gives a magnified image with the magnification V/U.

**Abbe sine condition**

Previously small angle approximation was used to derive the magnification factor. However Abbe realized that larger angles could be treated if \( \sin \theta / \sin \theta' \) remains constant. If

\[ \frac{\sin \theta_j}{\sin \theta'_j} = m \]

then,

\[ d'_j = \frac{\lambda}{\sin \theta'_j} = \frac{m \lambda}{\sin \theta_j} = m d_j \]

so the same result is obtained.

**Resolution limit of optical instruments**

Let's use the Abbe theory of image formation to find how aperture stop and coherence of illumination limit the resolution of imaging. The most well-known resolution criterion is one from Rayleigh and it applies to the self-luminous or incoherently illuminated object, such as starts (telescope imaging) or fluorescent objects (microscopy). For a single point, we can observe the Fraunhofer diffraction pattern (Fig. 2) of the aperture stop and it is called the point spread function. An extended object is a collection of point objects so the final image is the sum of the point spread functions of point objects. Thus the image is the convolution of the object intensity and the point spread function.

Fraunhofer diffraction limit gives the amplitude of diffraction at the point \( P(u,v) \)

\[ \Psi_p = \Psi(u, v) = \exp(ik_0 \overline{OP}) \iint f(x, y) \exp[-i(ux + vy)] \, dx \, dy \]

Where \( f(x,y) \) is the mask function of the object. The intensity becomes
$$I = |\Psi_p|^2 = |\exp(i k_0 \overline{OAP})|^2 \iint f(x,y)\exp[-i(ux + vy)] \, dx \, dy|^2$$

Thus the phase term \(\exp(i k_0 \overline{OAP})\) is irrelevant for measuring intensity. For the case of incoherent sources, considering the intensity is good enough to study the diffraction pattern, but for coherent sources the amplitude \(\Psi_p\) has to be under consideration. Let’s consider the case of incoherent point sources firstly. For a circular hole of radius \(R\), polar coordinates are handy to use. Set

\[x = \rho \cos \theta, \quad y = \rho \sin \theta, \quad u = \zeta \cos \phi, \quad v = \zeta \sin \phi\]

Then the amplitude equation becomes

\[
\Psi(u, v) = \exp(i k_0 \overline{OAP}) \iint f(x,y)\exp[-i(ux + vy)] \, dx \, dy
\]

\[
= \exp(i k_0 \overline{OAP}) \int_0^R \int_0^{2\pi} \exp(-i \rho \zeta \cos(\theta - \phi)) \rho d\rho d\theta
\]

\[
= \exp(i k_0 \overline{OAP}) \pi R^2 \frac{2J_1(\zeta R)}{\zeta R} = \Psi(\zeta, \phi)
\]

Where \(J_1\) is the Bessel function (Fig. 3).

In case of incoherent sources, the phase term \(\exp(i k_0 \overline{OAP})\) would cancel out the amplitude, so amplitude will not matter when we consider the diffraction. Thus only the intensity will matter and if we ignore the multiplication constant \(\pi R^2\),

\[
I(\theta) = \Psi^2 = [2J_1(\frac{1}{2} k_0 D \sin \theta)/(\frac{1}{2} k_0 D \sin \theta)]^2
\]

Where \(D\) is the diameter of the aperture, \(\theta\) is the diffraction angle and \(\zeta R\) is rewritten as \(\frac{1}{2} k_0 D \sin \theta\). Rayleigh considered if the central maximum of one lies outside of the first minimum of the other, two points are distinguishable. Since \(J_1(3.83) = 0\) as the first zero,

\[
\frac{1}{2} k_0 D \sin \theta_1 = \frac{\pi D \sin \theta_1}{\lambda} = 3.83 \approx \frac{\pi D \theta_1}{\lambda}
\]
this gives the minimum angular separation

\[ \theta_{\text{min}} = \theta_1 = \frac{3.83\lambda}{\pi D} = 1.22\lambda / D \]  
(Fig. 4, Top-Left).

This is a well-known resolution criterion. An alternative criterion is Sparrow criterion which is based the superb capability of human eyes to distinguish based on intensity differences. This considers two points distinguishable if their joint intensity function has a minimum in the middle of them. Thus when \( \theta = \theta_{\text{min}}/2 \) (at the middle of the separation of the emissions),

\[ \left( \frac{d^2 I}{d\theta^2} \right)_{\theta=\theta_{\text{min}}/2} = 0 \]

, which gives \( \theta_{\text{min}} = 0.95\lambda / D \) (Fig. 4, Top-Right).

These criteria become invalid when we consider the case when the two emissions are coherent. In this case since the phases match, so \( \exp(ik_0 \overline{OAP}) = 1 \), the amplitudes should be considered as

\[ A(\theta) = |\Psi| = 2J_1\left(\frac{1}{2}k_0 \text{ D sin } \theta\right) / \left(\frac{1}{2}k_0 \text{ D sin } \theta\right) \]

. The Rayleigh criterion would give the same result as before, but the points are not resolved as seen in the figure.

However Sparrow criterion can give a resolvable distance as \( \theta_{\text{min}} = 1.46\lambda / D \). This is higher than incoherent case due to the constructive interference of diffracted light (Fig. 4, Bottom-Right). Thus in general incoherent point sources result in better resolution.

However in general most microscopes work with coherent or partially coherent illumination, especially with lasers. Thus Abbe theory can be applied to coherent illumination. For the periodic model described in the Fig…, the first order appears at the imaging plane at angle \( \theta_1 \) is

\[ \sin \theta_1 = \lambda / d \]

To image an object with the period \( d \), the angular semi aperture \( \alpha \) has to be bigger than \( \theta_1 \) (Fig. 5).
Since
\[ d = \frac{\lambda}{\sin \theta_1} \]

The smallest period that can be imaged for this system is
\[ d_{\text{min}} = \frac{\lambda}{\sin \alpha} \]

If the objective is in an immersion oil having the refractive index \( n \), then the wavelength becomes \( \lambda/n \). Thus,
\[ d_{\text{min}} = \frac{\lambda}{n \sin \alpha} \]

So far we assumed the illumination is parallel to the axis, so that the 0\(^{\text{th}}\) and two 1\(^{\text{st}}\) orders (\( \theta_1 \) and \( \theta_2 \)) (Fig. 6A).

However the period can be imaged by the 0\(^{\text{th}}\) order and a single 1\(^{\text{st}}\) order. Thus the resolution can be improved if the illumination has an angle \( \alpha \) so that these two rays can get into the lens (Fig. 6B).

This can be viewed as the rays are hitting the sample of diffraction grating whose spacing is \( d \cos \alpha \). This gives
\[ d_{\text{min}} \approx d_{\text{min}} \cos \alpha = \frac{\lambda}{n \sin 2\alpha} \approx \frac{\lambda}{2n \sin \alpha} \equiv \frac{\lambda}{2NA} \]

, where NA stands for numerical aperture. This final result,
\[ d_{\text{min}} = \frac{\lambda}{2n \sin \alpha} \]

is Abbe’s law of diffraction limit, and it has been considered the ultimate resolution limit. For example, Alexa Fluor® 647 dye that was commonly used in this report, has its emission wavelength peak at 669nm. Thus, even with a high quality objective of NA=1.4, the resolution limit is around 240nm. In order to accomplish the best resolution, the sample is usually illuminated isotopically with a light cone having semi-angle of at least \( \alpha \) to get high resolution in all directions (Fig. 7).

**Attempts to overcome the resolution limit**
Localizing single molecules with high precision

The image of an isolated fluorophore would appear as diffraction limited broad peak of several hundred nanometers in width, its position can be precisely localized by determining the center of its emission pattern. It has been shown that a two-dimensional Gaussian function is the best fit to images of a single fluorescent dye, referred as a point spread function (PSF). The standard error of the mean (SEM) of the PSF is a measure of localization and it can be made small by collecting more photons and minimizing the noise factors \[10, 11\]. The precision of this localization process is given by:

\[
\sigma_\mu = \sqrt{\frac{s^2}{N} + \frac{a^2}{12N} \left( \frac{8\pi s^4 b^2}{a^2 N^2} \right)}
\]

where \(s\) is the standard deviation of the Gaussian distribution, \(a\) is the pixel size, \(b\) is the background and \(N\) is number of collected photons. The technique that the position of a single fluorophore can be determined with a precision as high as ~1nm was referred as Fluorescence Imaging with One Nanometer Accuracy (FIONA) \[12\].

However FIONA itself does not directly translate into image resolution of many fluorophores with close proximity. The emission patterns of multiple fluorophores can overlap with each other. If they are separated from each other by a distance less than the twice of PSF width, they are not resolvable by FIONA (Fig. 8A, B). Here the PSF width can be identified as the full width half maximum (FWHM) of the point spread function of a fluorophore, as Sparrow separation shows (Fig. 4, right panels).

Super-resolved single fluorophore microscopy

In order to be able to localize multiple fluorophores that are in close proximity, one could exploit use of photoswitchable fluorophores: molecules that can be switched between a dark and a bright fluorescent state, which can be controlled by light. The fluorescent states of these probes can be controlled in a way that ensures at any point in time, only a sparse subset of the fluorophores in the sample is in the bright state. As long as the images of the single molecules are not overlapping, each molecule can be localized with high precision. The molecules, whose positions have been determined, now can be switched off to the dark state, allowing activation of a new
subset of molecules that can be imaged and localized. By repeating this cycle of activation, localization, and deactivation, the positions of numerous closely spaced fluorophores may be determined by combining localizations of detected fluorophores. (Fig. 8C) [7].

The imaging process consists of many cycles of activation, imaging and deactivation of fluorophores. The density of the activated molecules depends on the intensity of the activation light. Shining a weak activation light activates a low density of fluorophores at each imaging frame. If there are two few fluorophores activated, stronger activation light is used to activate more at a time, but the density of the fluorophores should be kept low enough to prevent overlapping.

**Photoswitchable Fluorescent Probes**

A fluorescent molecule is called a photoswitchable switch when its spectral characteristics can be switched between different states under the control of an external light source. Several fluorescent proteins and organic fluorophores have been shown to display photoswitching behavior. For example, yellow fluorescent protein (YFP) cycles reversibly between a fluorescent and a dark state by alternating exposure to blue and violet light [13]. Similar behaviors were also observed with organic fluorophores, such as the red cyanine dye Cy®5, which can be reversibly converted between a fluorescent state and a dark state. Photoswitchable fluorophores have found increasing application for super-resolution imaging. A wide variety of photoswitchable synthetic fluorophores and fluorescent proteins are now available in many wavelengths of light [14, 15].

We use Alexa Fluor® 647 (A647), Alexa Fluor 568® (A568) and Cy®7 (Cy7) dyes for imaging, and Alexa Fluor® 405 (A405) for activating the imaging dyes from the dark state. The photoswitching behavior of these dyes is dependent on the presence of a thiol-containing molecule in the imaging solution such as β-mercaptoethanol (βME) or β-mercaptoethyamine (MEA, or referred as cysteamine) [14], or alternatively, with phosphine tris(2-carboxyethyl)phosphine TCEP [16]. When red and yellow lights are shined onto A647 and A 568 dyes respectively, they are initially fluorescent before switching into a dark state. When they are then exposed to UV light (405nm), a significant fraction of the molecules rapidly return to their fluorescent state. The reactivation can be made significantly more efficient when these dyes are paired, in close proximity, with a second fluorophore that absorbs UV light, such as A405.
Alexa Fluor 647 and Alexa Fluor 568 dyes are termed as reporter dyes, as they are imaged to reveal the super-resolution structure. Alexa Fluor 405 is termed activator dye, as it is used to reactivate the reporter dyes. We then pair Alexa Fluor 647 with Alexa Fluor 405 to make an optical switch which is turned off with red laser (647 nm) and turned on with violet laser (405 nm). When we do two-color imaging, we pair Alexa Fluor 568 with Alexa Fluor 405 to make another spectrally distinct optical switch, which is turned off with yellow laser (647 nm) and turned on with violet laser (405 nm).

**Instrumentation**

The instrumentation is described in Figure 9. We use Olympus IX-71, a standard inverted fluorescence microscope body. The microscope is equipped with a 100X NA 1.4 oil immersion objective that allows for both epi-fluorescence and objective-type total internal reflection fluorescence (TIRF) illumination schemes.

The imaging and the activation lasers are combined into a single beam using dichroic mirrors, which is then expanded by 7.5 times, and collimated using a pair of achromatic lenses. The collimated beam is focused on the back focal plane of the objective by using an achromatic lens. This lens is mounted on a translation stage, which allows adjustment of the incidence angle of the illumination light. For imaging cells, we set the incidence angle to near the critical angle of total internal reflection. This scheme allows illumination of several micrometer of thickness into the sample, while reducing the background signal from the imaging buffer, free floating probes, or fluorescence signal from a thick specimen. For imaging thin samples, incidence angle can be adjusted for a narrower depth of illumination so that imagine can be done with minimal background signal [18, 19].

We use three lasers at 647, 568 nm, and 750 nm to image A647, A568 and Cy7 respectively. 647nm and 568nm lasers have a power output of 100 mW, while 750nm laser has the output of 300 mW. 750nm laser had extra wavelength of the light which caused high background signal for Cy7 imaging, so a laser clean up filter was installed in front of it. Further details of the lasers, dichroic beam splitters and filters in the excitation path are listed in Table 1 and 2. We use an additional weak (less than 10 mW) laser at 405 nm for activation of the photoswitchable fluorophore pairs that use Alexa Fluor 405 as an activator fluorophore. Laser intensities are
controlled by passing the laser through a half-wave plate followed by a polarizing beam-splitter cube. Rotating the wave plate rotates the polarization, which, in turn, modulates the amount of light passing through the beam splitter. Neutral density filters are also used to further control laser intensities. Electronically controlled laser shutters are used to switch the illumination between the imaging and the activation lasers, in synchrony with the data acquisition.

The emission light is passing through bandpass filters designed to collect fluorescence from either A647, A568, or Cy7, and notch filters designed to block 647 and 568 nm laser lines. The details of the dichroic beamsplitters and emission filters are listed in Table 3. Then there are two achromatic relay lenses of focal length of 100mm and 150mm to give extra magnification of the sample. Also a cylindrical lens of focal length 1m was inserted in between these two lenses to cause astigmatism of single fluorophores to detect their z-positions, so that three-dimensional (3D) super-resolution imaging is possible [20]. The emission signal then is detected on an EMCCD camera suitable for low-light fluorescence imaging to detect single fluorescent molecules. Because of the extra magnification, the final pixel size on the EMCCD corresponds to 100nm of the sample. The image is acquired by a home written data acquisition program (C++) and saved on the computer. The program also controls laser shutters in synchrony with the camera frames.

Because the data is collected over a long period of time, we encounter both lateral and axial drifts. The lateral drift is corrected for during the data analysis procedure. We use CRISP (Applied Scientific Instrumentation) that substantially eliminates focus drift. The CRIFF is based on an LED IR light (850 nm) which passes through the objective lens, being reflected off the sample-coverglass interface, and then projected onto a photodetector. The position of the beam on the photodetector reports any relative motion between the objective lens and the sample, and this signal is fed back to a piezoelectric objective (PI), which corrects for the motion (Fig. 10).

**Sample Preparation**

We prepare samples for super-resolution imaging using immunofluorescence. Immunofluorescence technique uses the specificity of antibodies to their antigens to label fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualization of the abundance and distribution of the target molecule in the sample [1].
Our immunofluorescence protocol involves aldehyde fixation of the sample to preserve the cell morphology by cross-linking proteins. Cells are then treated with a mild detergent or enzyme to dissolve small holes in the membranes, so the antibodies could have access to the cytoplasm. We improve staining by blocking the non-specific binding of the antibodies to the specimen. The sample is then incubated firstly with the unlabeled primary antibody that specifically binds the target molecule, and then with the dual-labeled secondary antibody that binds to the primary antibody. The immunofluorescence protocol for mammalian cells is different from one for bacterial cells. The specific protocols are described in the next chapters (Ch. 2, 3). Here we describe secondary antibody labeling protocol.

**Secondary Antibody Dual-Labeling Protocol**

- Prepare the antibody to 1 mg/ml in PBS or water.
- Prepare 1M of sodium bicarbonate solution in PBS.
- Mix the following for the reaction buffer:
  1. 50 μl of 1 mg/ml antibody solution
  2. 2.5~3 μg Alexa Fluor 405 NHS ester
  3. 0.5~0.6 μg Alexa Fluor 647 NHS ester (or Alexa Fluor 568 NHS ester, or Cy7)
  4. 5 μl of 10X PBS
  5. 6 μl of 1M sodium bicarbonate solution
- Incubate the reaction solution for 30 minutes at room temperature in dark.
- Purify Antibody using the Bio-Spin 6 columns, following the instruction manual from the manufacturer.
- Take absorption spectrum to determine labeling efficiency. The ideal ratio of dye/antibody is 0.5~0.7 for reporter dye (A647, A568 or Cy7) and 2~3 for activator dye (A405).

**Imaging Buffer**

**For A647 or A568**

Prior to imaging, the appropriate imaging buffer is added into the sample well. For the photoswitchable Alexa Fluor dyes (A647 or A568), we use a buffered solution of pH 8.0 that
contains 50 mM Tris, 10 mM NaCl, and 10% glucose. Then right before imaging, and catalase (219001, EMD Millipore, 909 unit/ml) and Pyranose Oxidase (P423, Sigma-Aldrich, 4.44 unit/ml final concentration) were put as the oxygen scavenging system. Also for photoactivation characteristics of dyes, a thiol compound was added. For samples with many fluorophores, cysteamine (M9768, Sigma-Aldrich) was put (10mM final concentration). For samples with less fluorophores, β-mercaptoethanol (BME, M6250, Sigma-Aldrich) was put (1% final concentration). Cystemamine is more effective than BME to bring the fluorophores to the dark state, but the recovery rate reactivation is also low with cysteamine. Therefore it is only suitable with very bright sample, or samples with too much signal from background or floating probes.

To prepare imaging buffer of 500 µl:

Mix:

- 5 µl of βME
- 1 µl of catalase
- 3 µl of pyranose oxidase solution
- Base imaging buffer to a final volume of 500 µl

Base imaging buffer:

- 10 mM sodium chloride (NaCl), 50 mM Tris pH 8.0, 10% (weight/volume) glucose are mixed, then filtered by 220nm filtering disk to prevent contamination. The base buffer can be stored either in the room temperature or -20°C.

Pyranose oxidase stock:

- Dissolve 5 mg pyronase oxidase in 250 µl PBS (20mg/ml = 740 unit/ml) and store in 4°C.

Catalase stock:

- 454.5 (kilo units)/ml, stored in 4°C
For Cy7

Cy7 dyes appear much dimmer than A647 or A568 on EMCCD under the same imaging buffer condition as above. To let it emit more photons while maintaining its photoactivation characteristics, we used TCEP based buffer. The base buffer contains 0.1 M Tris, 12.5 mM TCEP, 2.5% glucose, 0.5mM ascorbic acid and 0.5mM methyl viologen [16]. They are all mixed in deuterium oxide (D2O) for yield maximum photon numbers from Cy7 [21].

Data Collection

During the experiment, a series of images is recorded, showing individual fluorophores switching on and off in time. We record 20-40 thousand image frames, 256 × 256 pixels each, at a 30 ms frame rate.

The procedure of data collection is as follows (Fig. 11):

- **Switch the fluorophores off:** We briefly shine imaging laser to the sample to send the fluorophores to the dark state.
- **Activate a subset of fluorophores:** We expose the sample to a weak activation laser for 30 milliseconds, which causes some of the fluorophores to switch to the fluorescent state. The intensity of the activation laser is adjusted to control the density of fluorophores activated. As the experiment proceeds and some fluorophores are permanently photobleached, the activation light intensity may be increased to maintain the density of activated fluorophores.
- **Image the fluorophores and switch them off:** We send the imaging laser to the sample to image the activated fluorophores. The density of the activated single fluorophores is low enough, such that their images do not overlap. We take 3 sequential images of the activated fluorophores. This takes long enough time for these molecules to switch off before the next activation frame. The intensity of the imaging laser can be adjusted to make sure all fluorophores switch off before the next activation frame.
- **Activate a new subset of fluorophores and image.** Activation of a subset of fluorophores and imaging them constitute one imaging cycle. We repeat the cycle for 20-40 thousand times.
• **Image the second color.** We first image Alexa Fluor 647 channel, for which we use red laser (647 nm) to image, and violet laser (405 nm) to activate. After imaging the Alexa Fluor 647 channel for 20-40 thousand frames, we move onto the Alexa Fluor 568 channel, for which we use yellow laser (568 nm) to image, and violet laser (405 nm) to activate.

**Data Analysis**

The data analysis algorithm is based on Mark Bates’ IDL code which was previously described [7]. We modified the original code to handle multi-color and 3D images.

The raw data is composed of many cycles of frames. One cycle contains one activation frame and three imaging frames. As the activation laser does not excite reporter dyes, the activation frames contain no signal. Therefore, activation frames are not taken into account in the data analysis.

**Peak Identification and Fitting**

The first task is to identify single molecule images and precisely determine their positions by fitting. In order to get rid of noises from various sources such as the EMCCD sensor or ambient lights, we take the single molecule images through blurring by Gaussian convolution of 9X9 pixels.

*Identification Step #1:* A single molecule image is several folds brighter than the background. To identify single molecules, a user defined intensity threshold (usually ~4) is introduced. In order to get the average fluctuation of the signal and noise in individual frames, we calculate the standard deviation of the intensity in that frame. Then, we find all the pixels whose values are greater than the multiplication of the threshold and the standard deviation of the frame.

*Identification Step #2:* Next we find the local maximum intensity pixels whose intensity value is greater than its 24 surrounding pixels. The local maximum intensity pixel would represent the intensity peak of a single molecule, whose image spans 25 pixels.

*Identification Step #3:* Some of the local maximum intensity pixels could come from multiple molecules whose images overlap, or bright non-specific signals on the surface. We get rid of
them by applying two filters based on sharpness and roundness of the image. Sharpness is defined as the intensity ratio between the peak and the background. Bright, non-specific signals typically have much higher sharpness value than single molecules, so we can filter them out with by applying the sharpness filter. Roundness is defined as the ratio between x variance and y variance in 9X9 pixels. Overlapping images of more than one single molecules are usually not as round as single molecule images. Therefore applying this filter eliminates images of multiple molecules (Fig.12).

**Fitting and Identification Step #4**: For images that passed the sharpness and roundness filters, a square region of 19X19 pixels surrounding local maximum intensity pixel is fitted with an Elliptical Gaussian function.

\[
G(x, y) = h \exp \left( -2 \frac{(x - x_0)^2}{w_x^2} - 2 \frac{(y - y_0)^2}{w_y^2} \right) + b
\]

Where \( b \) is the background level, \( h \) is the amplitude of the peak, \( w_x \) and \( w_y \) are elliptical widths, \( x_0 \) and \( y_0 \) are the center coordinates of the peak. If the software fails to fit any 19X19 square region of pixels with a Gaussian function, then that region is discarded.

During data analysis, the z-positions of the molecules are determined by comparing their \( w_x \) and \( w_y \) values to a calibration curve, that we generated from distributions of \( w_x \) and \( w_y \) values of images of fluorescently labeled single antibodies, as we moved the objective in z direction (Fig. 13).

**Trail Generation**

A single fluorophore can blink multiple times, appearing in consecutive frames, which may lead to double counting and less precision in the final image. To avoid this, we connect molecules that appear in consecutive frames by generating trails. If a trail is longer than 10 frames, it is discarded since it may come from non-specific signals which do not photobleach. After trails are generated, further filters (roundness, sharpness, maximum ellipticity and photons detected) are applied on each peak in every trail, to remove unqualified peaks. Then, for each trail, the final localization of the molecule is calculated from the average of center positions of the peaks within the trail, weighted by photon numbers in each frame.
**Drift Correction**

Any kind of drift such as vibration or thermal expansion would blur the final image since each image at each frame would shift over time. Z-drift is prevented in real time by a commercial feedback system called, ASI CRISP. The horizontal drift is corrected during data analysis. There are two methods we use.

*Drift Correction by fast Fourier transformation:* We divide the dataset with equal number of frames, and get sub-super-resolution images that look similar to each other, except for the effect of drift. We, then apply fast Fourier transformation (FFT) on each sub-super-resolution image. By comparing the center images in each transformed image, we determine the relative drift among sub-super-resolution images. By linear interpolation, we correct for the drift through all the frames (Fig. 14).

*Tracking a fiducial marker:* Another way to correct for the drift, is to track the position of a fiducial marker, such as a nano-diamond, during data acquisition. The change in the position of the marker is subtracted from each frame, yielding a drift-corrected image.

**Super-resolution Image Rendering**

The final super-resolution image is generated by plotting each localization as one point. These points can be either cross shaped markers or Gaussian rendered peaks. The size of rendered peaks would be proportional to the photon numbers of localizations.

*Color options:* The localizations in the final super-resolution image can be plotted in different colors based on their z values or their frame numbers. Coloring based on their z values is informative about the z-positions of localizations when they are plotted in 2D, while coloring based on frame number reveals when each localization was detected.

*Two color visualization:* After generating a super-resolution image, the software creates a storage file that includes the coordinates, photon number and the color of each localization. We generate separate storage files for each channel. Multiple storage files for different colors is combined together to generate multi-color images.
Generating a far-filed image: The software generates a conventional diffraction limited far-field image to compare to the super-resolution image, by summing up all the frames of the raw data. Since each frame contains stochastic subset of the total fluorescence, summing them all up would give a total fluorescence image. The software subtracts a certain background value from each frame before summing up, since otherwise the background accumulates and overwhelms the signal. Drift correction is also applied during generation of diffraction limited image. Alternatively, a far field image can be generated directly from a super-resolution image, by rendering each localization peak to be diffraction limited, that is, each one’s FWHM would be setup around 250nm. These two ways of making far-field images are comparable. (Fig. 15).

Exemplary Applications of Super-Resolution Imaging

Super-resolved single fluorophore microscopy has been used to various examples ranging from bacterial cells as well as eukaryotic cells. In the following chapters (Ch2~4) we will discuss the unpublished new findings by this super-resolution imaging technique. In this section, we introduce the standard samples that well mainly used for testing super-resolution techniques, and also antiviral response related proteins for further demonstrations.

Standard examples and two-color imaging

Microtubules are found in the cytoplasm of eukaryotic cells and carry out a variety of functions such as transport or structural support. To image microtubules, BSC-1 (Kidney Epithelial Cells of Monkey Origin) cells or MEF (Mouse Embryonic Fibroblasts) cells were fixed and immunostained with photoswitchable-switches (A647/A405 pairs) through indirect immunofluorescence targeting β-tubulin. Super-resolution images provided much more details (Fig. 15).

Clathrin is a protein that forms coated vesicles. To image clathrin coated vesicles, BSC-1 cells or MEF cells were fixed and immunostained with photoswitchable-switches (A647/A405 pairs) through indirect immunofluorescence. A conventional image of the clathrin coated vesicles is shown in Figure 16 with the corresponding super-resolution image. Compared to the diffraction limited blurry image, the super-resolution technique provides strikingly detailed structures (Fig. 16 C, D).
Microtubules and mitochondria: To demonstrate simultaneous imaging of microtubules and mitochondria, BSC-1 cells or MEF cells were fixed and immunostained with A647/A405 pairs to target mitochondria, and A568/A405 pairs to target β-tubulin (Fig. 17).

RIG-I: Upon viral infection, innate immunity of mammalian cells is triggered. It is composed by the cascade of antiviral signaling proteins. For double stranded RNA (dsRNA) virus, it starts from retinoic acid-inducible gene-I (RIG-I) protein which is a dsRNA sensor [22]. For certain types of viruses, RIG-I localizes in cytoplasmic granular aggregates called as antiviral granules (AVGs) [23, 24]. We transfected cells by polyinosinic:polycytidylic acid (poly I:C), a dsRNA analogue, to induce AVGs. We found that RIG-I forms clusters in AVGs (more details in Ch. 2). Immunostaining RIG-I with A647/A405 pairs provided an image of RIG-I clusters (Fig. 18), and we also used this system to further test other dyes for super-resolution imaging.

**Challenges in multi-color imaging and attempts to overcome them**

A647 has been observed to be the best reporter dye through our experience, and previous reports. On the other hand, A568, the other reporter dye we used for two color imaging, did not perform as well as A647 [14]. Microtubules labeled by A568/A405 have disconnected, inhomogeneous, interrupted and clustered structures (Fig. 17) compared to microtubules labeled by A647/A405. The quality of a reporter dye for super-resolved single-fluorophore microscopy depends on several properties such as on-off duty cycle, photostability, number of switching cycles, quantum yields and etc [14]. In short, A568 has a high duty cycle (the fraction of time a fluorophore stays in the on state), so that most of the A568/A405 switches do not go to the dark state in the beginning of the experiment, hindering effective detection of single localizations. Then later many of switches stay on, yielding artificial clusters. Also many of switches are not recovered to the fluorescent state once they switch off, even in the presence of strong UV laser, so that only fraction of the target structure is imaged. These issues of A568 dyes are closely related to the self-activation character of it, so it can be minimized by carefully controlling the labeling ratio to the targets, or samples with sparse and thin targets such as bacterial RNA. However for thick (several micrometers) and dense samples such as RIG-I clusters in AVGs, it was very challenging to get an image with a quality as good as with A647 labeling (Fig. 19C, left).
Thus we searched for an alternative dye that can replace A568, and we found Cy3B and Cy7 to be promising. Cy3B has shorter duty cycle, that is, Cy3B/A405 switches can switch off more effectively than A568. Also they could be effectively switched on and off for several thousands of image frames, but the activation effect by 405nm laser was not as sensitive as A568. Then with Cy3B we could obtain circular and hollow structures of clathrin, which we could never obtain by using A568 (Fig. 19). Cy3B was imaged using a 561nm laser (Cobolt, 100mW). However for thick samples such as RIG-I in AVGs, Cy3B still posed the issue of self-activation. Therefore we then tried the 2nd alternative dye, Cy7. Cy7/A405 switch has a comparable short duty cycle as A647/A405 and it can be effectively switched off by 750nm laser. However the critical issue Cy7 imaging has been its low signal on EMCCD, due to the emission power loss by the objective lens, lenses in the emission path, and low detection efficiency of EMCCD for infrared light. This leads each localization event in our imaging posed less accuracy, yielding low resolution image even after combining all the photoactivated localizations [14]. This issue has been overcome by various techniques such as using TCEP instead of thiols [16] and adapting D2O instead of water [21] for imaging buffer. Then we could get several folds brighter localization events for Cy7/A405, and could obtain comparable images to A647/A405. Cy7/A405 showed clear advantage over A568/A405 for various systems such as mitochondria (Fig. 19B) or RIG-I in AVGs (Fig. 19C). Mitochondria images obtained by Cy7/A405 was comparable to images obtained by A647/A405 (Fig. 17B), and RIG-I images obtained by Cy7/A405 was comparable to images by A647/A405 (Fig. 18B). We then attempted to obtain two-color images by employing A647/A405 and Cy7/A405 together. Microtubules labeled by A647/A405 and mitochondria labeled by Cy7/A405 showed much more connected structure than by using A568/A405 as the second switch (Fig. 20A, B, Fig. 17C). Also colocalization studies become available. As a positive control, we labeled RIG-I by one set of 2nd antibody labeled with Cy7/A405, then labeled RIG-I again by another set of 2nd antibody with A647/A405. Then we could obtain certain level of colocalization (Fig. 20C). This sample serves as a positive control for colocalization. Even targeting the same protein RIG-I, the colocalization efficiency is not 100%, maybe due to labeling efficiency or chromatic aberration. Then we set this as our reference as the positive control. Then we immunostained poly I:C by antibody against dsRNA (Scicons, K1). Poly I:C labeled by Cy7/A405 and RIG-I labeled by
A647/A405 showed similar level of colocalization, supporting the idea that RIG-I is a dsRNA sensor (Fig. 20D).

In conclusion, we demonstrated Cy3B and Cy7 could be attractive alternatives to A568, and they will enable three-color super-resolution imaging with A647.
Figures and Tables

Figure 1. Formation of the image of a diffraction grating

(A) Five orders of diffraction are depicted as solid lines. They produce five foci $S$ in the focal plane $F$. The angular semi-aperture of the lens is $\alpha$. (B) Two grates ($O_1$ and $O_2$) go through different paths, but end up hitting the same spot in the image plane. In the same principle, the image will be composed of interference pattern.
Figure 2. Illustrating Fraunhofer diffraction by two-dimensional object. The object plane is not parallel to the lens in general.
Figure 3. (A) The radial amplitude distribution in the diffraction pattern of a circular aperture is function $J_1(x)/x$. (B) Fraunhofer diffraction of circular hole.
Figure 4. The effects of incoherency and coherency of the illuminations in the case of adding images of two pinholes, in two distances defined by Rayleigh separation or Sparrow separation.

(Top-Left, Top-Right) When the pinholes are incoherently illuminated, both Rayleigh separation and Sparrow separation can make the two images of pinholes separable.

(Bottom-Left) When the pinholes are coherently illuminated, Rayleigh separation fails to make the two images of pinholes separable.

(Bottom-Right) When the pinholes are coherently illuminated, Sparrow separation can make the two images of pinholes separable. Here the Sparrow criterion is $1.46\lambda/D$ which is bigger than $0.95\lambda/D$ the Sparrow criterion of incoherent illumination.

(Right panels) For a case of if the point spread function of one image can be fitted with 2-dimensional (2D) Gaussian function, Sparrow separation can be considered as the full width half maximum (FWHM) of the 2D Gaussian function.
Figure 5. To image an object with the period $d$, the angular semi aperture $\alpha$ has to be bigger than $\theta_1$, the first order diffraction angle.
Figure 6. Abbe’s theory of resolution

(A) The 0th order and two 1st orders only can constitute the image of a diffraction grating, yielding the resolution to be $d_{\text{min}} = \lambda / (n \sin \alpha)$.

(B) The 0th order and only one 1st order can constitute the image of a diffraction grating too, and these are the minimal components for forming the image. This yields the resolution to be $d_{\text{min}} = \lambda / 2(n \sin \alpha)$, Abbe’s diffraction limit.
Figure 7. Conical illumination of a specimen to get the highest optical resolution
Figure 8. Conceptual schematics of super-resolved microscopy (A) If two fluorophores are apart by more than one’s point spread function (PSF) width, they are resolvable and their localizations can be obtained with high precision by Fluorescence Imaging with One Nanometer Accuracy (FIONA). (B) However if they are part by less than PSF width, they are not any more resolvable. (C) Super-resolved single fluorophore microscopy is done by collecting many frames of images, where stochastic activation and imaging of only small subset of fluorophores are images at each frame. Fluorophores that are apart more than their PSF width can be analyzed to give precise localizations that eventually are collected to generate the final super-resolved image.
**Figure 9.** A schematic of the super-resolved single fluorophore microscope setup.

DM: Dichroic mirror, EF: Emission filter, NF: Notch filter, HW: Half-wave plate ($\lambda/2$), L1-3: Achromatic Lens, M1-5: Mirror, Obj: Objective lens (NA 1.4), P: Periscope, PBS: Polarizing beam-splitter, S: Shutter (Uniblitz LS6T2), TL: Tube lens, CL: Cylindrical lens (f=2m, SCX-50.8-1000.0-UV-SLMF-520-820 from CVI Melles Griot), ND: Neutral density filter (NE01B-B, NE02B-B, NE03B-B, NE04B-B for 750nm laser), GND: Gradient neutral density filter, OFC: Optical fiber coupling (Thorlabs M31L01, SM1FC, CP02, AC050-008-B-ML), LCF: Laser clean-up filter (Chroma, ZET750/20x), CRISP: Continuous Reflective Interface Sample Placement, ASI), Ctr: Controller

The microscope is based on an inverted microscope. Laser intensities are controlled by passing the laser through a half-wave plate (HW) followed by a polarizing beam-splitter (PBS) cube. Different laser lines are combined into a single beam using dichroic mirrors (DM). The beam is then expanded 10 times by lenses L1 and L2 and focused on the back focal plane of the objective (Obj) using lens L3. L3 is on translation stage, which allows adjustment of the incident angle of illumination. The light is detected on the charge-coupled device (CCD) camera after passing through a set of bandpass filters (BF) to collect emission from specific fluorophores, and notch filters (NF) to block laser lines. A cylindrical lens (CL) is used in emission path for astigmatism imaging in 3D super-resolution imaging. Lenses L4 and L5 are used to magnify the image for an additional 1.5 times. ASI CRISP is used as a focus-lock system (Fig. 10).
Figure 10. The principle of ASI CRISP. CRISP is installed at the C-mount of the microscope, between the microscope body and the camera. IR light (850nm) from LED goes through the internal optics of CRIPS, then hits the sample and reflects back due to the index mismatch ($n_1$=the reflective index of glass, $n_2$=the reflection index of water based imaging media around the cell to be imaged). The reflected beam then follows a different path than it came from, and hits the photo detector (upper boxed drawing). To ensure this configuration of beam path, only one half of the objective pupil is illuminated. Once axial drifts occur, the reflected beam follows a different path than previously, hitting different spots on the detector (lower boxed drawing). Therefore CRISP can correlate the relative axial location of the sample to its detector reading.
**Figure 11.** The imaging scheme of a two color super-resolution experiment. Firstly, red laser is sent to the sample to turn off the Alexa Fluor 647 dyes. Violet laser is then briefly applied to switch some of the fluorophores to the fluorescent state. We send the red laser to the sample to image the activated fluorophores in 3 sequential frames. This is one imaging cycle. We repeat the cycle for 20–40 thousand times. After imaging the Alexa Fluor 647 channel, we move onto the Alexa Fluor 568 channel to take another for 20–40 thousand frames, for which we use yellow laser for imaging, and violet laser for activation.
Figure 12. An image frame taken from a series of super-resolution imaging frames, where optical switches are A647/A405 pairs. (A) The bright spots in the image correspond to the fluorescence emission from single fluorophores, which do not overlap. (B) An automated peak finding algorithm is used to identify peaks. The identified peaks are marked by white circles.
Figure 13. 3D super-resolution imaging (A) Calibration curve generated from distributions of $w_x$ and $w_y$ values of images of fluorescently labeled single antibodies, as the objective is moved in z direction. (B) An example of 3D imaging. Microtubules are immunostained by A647/A405, and they form a sea-shore like gradual height distribution.
Figure 14. Correction of lateral drift in super-resolution images. (A) A super-resolution image before drift correction (Green spots correspond to β-tubulin, violet spots correspond to Tom20 in a mammalian cell). (B) Corresponding super-resolution image after drift correction. The data set is divided into a number of equal time segments. Sub-super-resolution image for each segment is generated. By calculating the correlation function between each sub-super-resolution images generated from consecutive segments, the drift can be determined for that time period. The image can be then corrected by subtracting the drift from localizations. (Scale bar=2 μm)
Figure 15. Generating far-field images can be done by (A) adding all the frames together, or (B) rendering each final localization to be diffraction limited. (C) The corresponding super-resolution image for (A), (B). Scale bar = 1 μm. (D–G) Another example of far-field image generated by rendering each localization to be diffraction limited. Zoomed images (F and G) are from the white boxes (D and E). All the examples are β-tubulin images of mammalian cells, labeled by A647/A405 pair.
Figure 16. Super-resolution images clathrin-coated vesicles (A and D) with diffraction limited conventional images (A and C). Zoomed images of the white squares (C, D) show striking contrast between the conventional image (C) to the super-resolution image of closely placed three circular and hollow clathrin vesicles. Note the pixel size of the conventional raw image is 100nm (C), twice of the scale bar of the super-resolution image (D).
Figure 17. Two color super-resolution imaging of (A) microtubules labeled by A568/A405 and (B) mitochondria labeled by A647/A405, and the combined image (C).
Figure 18. RIG-I in an antiviral granule (AVG) in the cytoplasm of an MEF cell. (A) Conventional image of an AVG, 12 hours after poly I:C transfection. (B) the corresponding super-resolution image.
**Figure 19.** Comparisons of A568 with alternative dyes. (A) An image of clathrins labeled by A568/A405 shows irregular structure (left), while Cy3B/A405 gives a circular and hollow structure (right). (B) An image of mitochondria labeled by A568/A405 shows artificial clusters (left), while Cy3B/A405 labeling (right) gives a uniform mitochondria image. (C) An image of RIG-I in an AVG (circled) shows dispersed and irregular structure, while Cy7 labeling (right) gives a well clustered structure.
Figure 20. Multicolor images by Cy7. (A) An image of microtubules (red, A647/A405) and mitochondria (violet, Cy7/A405) gives a more connected structures for mitochondria, being comparable to A647/A405 (Fig. 15). (B) A zoomed-in image. (C) A positive control for two-color imaging. MEF cells were transfected by poly I:C, a double strand RNA (dsRNA) analogue. RIG-I was labeled by two identical antibodies with A647/A405 (red) and Cy7/A405 (violet). Two channels show a certain level of colocalization. (D) RIG-I (Cy7/A405, violet) and dsRNA (A647/A405, red) show similar level of colocalization as the positive control (C). dsRNA was labeled by an antibody against it.
<table>
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<tr>
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<th>Near IR laser</th>
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<td>Crystalaser</td>
<td>Shanghai Dream</td>
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<td>Type</td>
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<td></td>
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<tr>
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<td>Direct, paired with HW&amp;PBS</td>
<td>Direct, paired with HW&amp;PBS</td>
<td>Multimode optical fiber coupled, paired with ND filters and a clean-up filter</td>
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Table 1. The list of lasers used in the setup.
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Table 2. The list of dichroic beamsplitters and filters used outside of the microscope body in the excitation path
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<th>(\text{Purpose})</th>
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<td>Imaging without ASI CRISP, without Cy7 imaging</td>
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<tr>
<td>Semrock</td>
<td>Di01- FF408/504/581/667/762-Di01-25X36</td>
<td>Allows near IR imaging as well as 850nm, but cuts some A568 signal</td>
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<td>Blocks beyond Cy7 emission signal range, transmits more signal for A568</td>
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<td>Allows near IR imaging as well as 850nm, but cuts some A568 signal</td>
<td>Imaging with ASI CRISP, or for Cy7 imaging</td>
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<tr>
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<td>Imaging A568 (blocking 568nm)</td>
<td>Imaging A568 (blocking 568nm)</td>
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<table>
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<td>Blocking 568nm and 647nm</td>
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<tr>
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<tr>
<td>Purpose</td>
<td>Additionally blocking 568nm</td>
<td>Blocking accidental incidence of 750nm laser</td>
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Table 3. The list of dichroic beamsplitters and filters used inside of the microscope body and the emission path.
References


Chapter 2

RIG-I Clusters in Granule under various Stress Conditions including Viral Infection

Abstract

Mammalian cells have the innate immunity as the first line of defense against viral infection, triggered by Toll-like receptors (TLRs) and retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) sensing viral RNA. Certain types of viruses cause RIG-I to localize in antiviral granules (AVGs), but no further spatial detail was reported due to the limitation of nano-scale imaging. To address this issue, we imaged RIG-I using three dimensional super-resolution microscopy, and observed RIG-I forming clusters in AVGs. We analyzed the data by cluster analysis method. Our data suggest that RIG-I is intrinsically clustered but double stranded RNA (dsRNA) or stress conditions stimulates formation of RIG-I clusters with increased number and size (around 86~117nm) in AVGs or stress granules (SGs). Also AVGs tend to be bigger than SGs, implying the structural differences between these granules.

Introduction

Cells are exposed to many external threats, and one example is viral infection. The innate immune response is the first line of defense, even though viruses often block it [1, 2]. If the response can continue without being inhibited, it eventually induces type I interferon (IFN) which activates antiviral proteins and interferes with viral replication [3, 4]. The activation of IFN is regulated by the cascade of antiviral signaling proteins. The cascade starts from retinoic acid-inducible gene-I (RIG-I) which is a double stranded RNA (dsRNA) helicase enzyme. RIG-I recognizes foreign dsRNA and initiates the cascade by activating mitochondrial antiviral signaling protein (MAVS). MAVS then activates transcription factors to induce interferon (IFN).
IFN then activates PKR that blocks eukaryotic initiation factor 2 (EIF2) activity. This leads to translation inhibition of viral gene, blocking the replication of virus. IFN also can send antiviral signals to other cells, or cause apoptosis if no other means of defense is available [5-7] (Fig. 21A). However some viruses can block this defense mechanism. For example, influenza can encode nonstructural one (NS1) protein that can block the antiviral signaling cascade pathways, so that INF induction is inhibited while influenza can replicate utilizing the translation pathway of the host (Fig. 21B). Therefore influenza virus lacking NS1 (IAVΔNS1) can be effectively blocked by the innate immune response (Fig. 21A).

RIG-I consists of two caspase activation and recruitment domains (CARD) at the N-terminus and a DExH/C motif helicase domain and carboxyl C-terminal domain (CTD). Two CARD domains are important for signal activation, and DExH/C motif helicase domain and CTD function as a viral RNA recognition unit [8].

Although RIG-I has not been found to be associated with any subcellular structure at steady state, it was reported that infection by Influenza A virus lacking NS1 (IAVΔNS1) causes RIG-I to form cytoplasmic granular aggregates which resemble stress granules (SGs) [1]. SGs are cytoplasmic ribonucleoproteins (mRNPs) that form as a response to stress. They are initiated by stress-induced phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α), leading sudden translational arrest and rapid polysome disassembly. In this process, mRNAs for translating “housekeeping” proteins are stalled and stored in SGs, from disassembling polysomes. However mRNAs for translating enzymes involved in damage repair is not recruited to SGs, and hence their translation is enhanced. SGs also typically contain 40S ribosomal subunits, eukaryotic translation initiation factors and other poly(A)-binding protein (Pabp). SGs are assumed to be sites of mRNA triage where individual mRNAs are sorted for storage, degradation, or translation during stress and recovery. Beyond this role, they also serve as hubs intercepting signaling molecules to communicate “state of emergency” to other signaling pathways such as metabolism, growth and survival [9-13].

To induce SGs in individual cells, drugs that stall translation initiation are usually used, but not all of such drugs cause SGs formation. Both pateamine A and cycloheximide stall translation initiation [11, 14, 15]. Pateamine A does not affect elongation of ribosomes, and it causes SGs formation. However, cycloheximide inhibits translational elongation of ribosomes, and it
prevents SGs assembly and forces the disassembly of preformed SGs [16]. This reveals SGs are dynamic structures. Without continuous influx of new translation initiation complexes, i.e. mRNPs released from polysomes, SGs disperse [15]. Most SG components exhibit very short (seconds to minutes) resident times while SGs remain to minutes to hours [13].

Recent studies suggested that the granules induced by viral infection may not be SGs because they do not contain 40S ribosomal subunit and do not disassemble under the treatment of cycloheximide [17, 18]. Therefore in this report we refer the granules formed by viral infection as antiviral granules (AVGs) to distinguish them from SGs.

SGs are ranging from 0.1 to 2 µm in the diameter [10], but due to the diffraction limit of light microscopy, no further details within SGs have been revealed. Previous in vitro studies suggested RIG-I forms oligomers under dsRNA infection/transfection [8, 19, 20], which has not been observed in vivo. In this report, we present RIG-I clusters in vivo by our super-resolved single-fluorophore microscopy (super-resolution microscopy for short). We discovered that RIG-I was intrinsically clustered, with the average diameter ranging 80~110nm depending on various conditions.

**Results**

**RIG-I localizes to granules upon dsRNA infection/transfection and various stress conditions**

To image cellular localization of RIG-I, we transfected MEF cells with polyinosinic:polycytidylic acid (poly I:C), a synthetic analog of dsRNA (double-stranded RNA) virus. 12 hours after transfection, we fixed the cells and labeled RIG-I by immunofluorescence, and imaged them under an optical microscope. For mock-treated cells, RIG-I is dispersed in the cytoplasm. However once the cells are transfected with poly I:C, a dsRNA analog, RIG-I exhibited speckle-like distribution in the cytoplasm. These speckles also contain T-cell restricted intracellular antigen-related protein (TIAR), a marker for SGs/AVGs (Fig. 2A). Next we subjected the cells to different stress conditions, such as infection with IAVΔNS1, sodium arsenite (NaAsO2) treatment, and heat shock, and in all of these cases RIG-I localizes to AVGs or SGs (Fig. 22B). These observations are in accordance with the previous reports [1, 21].
RIG-I forms clusters in AVGs

Next, we imaged the spatial organization of RIG-I within individual SGs by 3D super-resolution microscopy [22, 23]. Here we are comparing four groups of samples treated by different stimuli, and two control groups (Table 4). The first group is cells transfected by poly I:C. The second group is cells infected by IAVΔNS1. Often times these two groups would be referred to cells infected/transfected by dsRNA. The third group is cells with NaAsO2 treatment. The fourth group is cells with heat shock. These two groups are for observing SGs formed by stress. Then the first control group is cells without any treatment (mock), but stained for RIG-I imaging. The second control group is cells fixed and prepared for imaging, but without the primary antibody. This group serves as a negative control for nonspecific binding of 2nd antibodies to the cellular structures. The 2nd antibody used for this control is the same for imaging RIG-I, so it can be a direct comparison to samples for imaging RIG-I.

In contrast to the conventional images where RIG-I appears uniformly dispersed within AVGs, super-resolution images revealed clustering of RIG-I in SGs (Fig. 23). TIAR, on the other hand, does not display as much clustering as RIG-I (Fig. 23B, lower panel). In order to quantify clustering, we used a clustering algorithm to identify clusters based on the spot density around every spot of the data set [24] (Fig. 24C).

We identified clusters of RIG-I in different conditions to study the effect of dsRNA infection/transfection on RIG-I clustering. All the numbers obtained for this analysis are listed in the Table 4. We describe notable points in the following.

Firstly we obtained the percentage of clustering upon different conditions. Under poly I:C transfection or IAVΔNS1 infection, 58(±1, ±2 respectively)% of RIG-I localizes in clusters in AVGs (Fig. 25A, 7B left panel), while in mock-treated cells 48(±2)% of RIG-I localized in clusters (Fig. 25A), and in the control cells immunostained without primary antibody but only 2nd antibody, only 25(±2)% of RIG-I localized in clusters (Fig. 26A). Since the percentage of clustering is higher for RIG-I than the 2nd antibody control, RIG-I is intrinsically clustered. Then this clustering is enhanced by about 10% in AVGs under dsRNA (poly I:C or IAVΔNS1) infection/transfection.
Secondly we obtained sizes of clusters. The size of a cluster is defined by twice of the average distance from the center of a cluster to every point in the cluster, so it can be considered as a “diameter” if a cluster is like a sphere. Even though RIG-I in AVGs and cytoplasm showed same percentage of clustering upon dsRNA infection/transfection, the former case showed 112(±2) nm (for poly I:C, Fig. 25B) or 107(±3) nm (for IAVΔNS1, Fig. 27B right panel) average size while the latter case had 77nm(±1) (Fig. 25B). On the other hand for the mock-treated cells, the average size of clusters is 93(±2) nm (Fig. 25B), a value between the cytoplasmic RIG-I and RIG-I in AVGs. This indicates that the intrinsic RIG-I clusters in intact cells change their sizes upon dsRNA infection/transfection, getting smaller in the cytoplasm and getting larger in AVGs.

We then conducted cluster analysis on TIAR, the SG marker, under poly I:C transfection. 15(±3)% (in AVG) or 11(±2)% (in the cytoplasm) of TIAR localizations are clustered, displaying much less clustering than not only RIG-I (Fig. 26A), but also less than 2nd antibody in the negative control (25(±2)% , Fig. 26A). In terms of the sizes, AVGs (85(±2) nm) and the cytoplasm (69(±2) nm) are notably smaller than RIG-I clusters, but bigger than 2nd antibody clusters in the negative control (62(±1)nm, Fig. 26B) This indicates that without target proteins, 2nd antibodies may form small clusters in the cell body, but in the presence of the target protein they relocate themselves around the target protein structures. When the size and percentage of each cluster are plotted against each other, RIG-I in AVGs shows distribution district from TIAR in AVGs (Fig. 26C).

**RIG-I clustering is independent of dsRNA**

To determine if the observed clustering of RIG-I is dependent on dsRNA infection/transfection, we treated the cells under stress conditions as sodium arsenite (NaAsO₂) exposure or heat shock. RIG-I formed clusters in SGs under both cases (53(±1)% and 56(±3)% respectively) (Fig. 27A, B). In these SGs, RIG-I cluster sizes are 106(±2) nm and 86(±2) nm respectively (Fig. 27C). When the size and percentage of each clusters are plotted together, RIG-I in AVGs/SGs in three cases (IAVΔNS1, NaAsO₂, and heat shock) have distinctive distribution from 2nd antibodies nonspecifically bound to the cells (Fig. 27D).
AVGs are bigger than SGs

Since our super-resolution microscopy could precisely determine the boundary of each AVGs/SGs, we measured the average size of each of AVGs/SGs under dsRNA infection/transfection, sodium arsenite treatment and heat shock. The average area of RIG-I and TIAR in AVGs induced by dsRNA (1.64 (±0.16) μm² for RIG-I, 2.7(±0.67) μm² for TIAR) turned out to be bigger than the case of SGs induced by NaAsO₂ and heat shock (0.84 ~ 1.21 μm²) (Fig 27A).

Discussion

RIG-I in AVGs/SGs forms clusters universally

We have presented data for RIG-I forming clusters in AVGs/SGs and cytoplasm under dsRNA infection/transfection and various stress conditions. Since this clustering was observed in every case, one may argue this may be an experimental or analytical artifact. Super-resolution microscopy based on photoswitchable fluorophores does have the possibility of making artificial clusters, and a way to distinguish real clusters from artificial clusters was suggested [25]. For our case we have TIAR clusters serving as the control. Both RIG-I and TIAR are labeled with Alexa Fluor® 647, but for TIAR we observed significantly less clustering (Table 4, Fig. 24B, C, lower panels, Figure 25A). They are labeled by different 2nd antibodies, but they are very comparable in terms of clustering (24(±1)% or 25(±3)% respectively for RIG-I or TIAR, Table 4), so this different clustering behavior of TIAR is not coming from the 2nd antibody clustering. As another control, we labeled RIG-I using a different 2nd antibody from a different manufacturer, and we could also observe clustering of RIG-I in AVGs (Table 4). To further confirm RIG-I clustering is not an artifact of our super-resolved single-fluorophore microscopy, we conducted structural illumination microscopy (SIM) and also could obtain RIG-I clustering, in both cytoplasm and AVGs under poly I:C transfection (Supplementary data). Collectively, these data suggest that it is less likely that RIG-I clustering is from experimental artifacts.

Since cluster analysis was done by an algorithm to identify clusters based on the spot density around every spot of the data set (more details in the Materials and Methods), one may suspect
artifacts from it may arise. It is possible, since we chose two parameters (N points and Eps for DNSCAN cluster analysis) by visual feedback. We tested several quantitative approaches to get the best set of parameters, and the best ones could effectively remove artificially detected clusters (Appendix, Fig. A16, A17), but they can possibly eliminate some real clusters too (Fig. A14A). This possibility that our choice may not be the best one would possibly contribute 15~26% of TIAR clustering AVGs (Table 4) even though TIAR distribution looks more uniform (Fig. 24C, 28B, lower panels). Also TIAR cluster size was measured 68~115 nm (Table 4) but it may be overestimated, since a uniform but dense area in TIAR case could be picked as a cluster by the software. The spontaneous blinking characteristics of A647 dyes can lead to such artifact, so we conducted further analysis to investigate the nature of clustering artifacts in some cases (Supplementary data). In short, RIG-I clusters in AVGs are mostly real, but cytoplasmic RIG-I clusters have contributions from artificial clustering, and TIAR clusters both in AVGs and cytoplasm are mostly artificial (Supplementary data).

On the other hand, one may argue against the universal clustering behavior of RIG-I in AVGs/SGs due to the smaller size (~86(±2) nm) of RIG-I clusters under heat shock (Fig. 26B, right panel). Even though the size is smaller than the dsRNA infection/transfection case and even similar to TIAR clusters, the heat shock case still gives ~56(±3)% of RIG-I localizations in clusters (Fig. 27B, left panel), significantly more than the case (15~26%) of TIAR in AVG/SGs (Table 4). Thus RIG-I clusters in the heat shock response would be real, but the reason why the size of them are smaller than other cases is uncertain.

RIG-I clustering was also observed without any treatment. Cells with mock-treatment showed 48(±2)% of clustering (Fig. 25A) which is lower than the case of poly I:C transfection in AVGs and cytoplasm (~58(±1, 2)% Fig. 24A) but much higher than TIAR case (11(±2)% in cytoplasm, 15(±3)% in AVGs, Fig. 25A). This suggests that RIG-I is intrinsically clustered, but the clustering is enhanced by dsRNA. Also the average size of RIG-I clusters in the mock-treated cells is between RIG-I clusters in AVG and cytoplasm. This suggests the intrinsic clusters of RIG-I go through dissolution/reassembly upon dsRNA while AVGs are formed.

There have been in vitro studies of how RIG-I is activated to initiate antiviral response. The binding of viral RNA to the CTD and helicase domains of RIG-I induces an ATP-dependent conformational change that exposes the CARDs [19], leading 2CARDs to assemble into
polyubiquitin mediated helical tetramer [8]. Then RIG-I assembles into filamentous oligomers along dsRNA [20]. Polyubiquitin mediated RIG-I oligomerization is essential to activate IRF3 and induce IFNβ [19]. These in vitro studies support the idea that RIG-I would also form oligomers in vivo, as our data suggest.

RIG-I relocalized into not only AVGs, but also SGs. The involvement of RIG-I in general stress response is not known yet, so why RIG-I localizes into SGs and how it forms clusters within are yet to be further investigated. We suspect there may be protein or nucleotide linkers to trigger cluster formation of RIG-I within SGs, so multicolor super-resolution imaging with colocalization study is much desired.

**AVGs are different from SGs**

In this report we presented that RIG-I and TIAR form AVGs under poly I:C transfection or IAVΔNS1 infection, and they form SGs under sodium arsenite treatment and heat shock. Even though AVGs and SGs all showed similar RIG-I behavior, the sizes (the area defined by image contrast) of AVGs are turned out to be bigger than SGs (Fig 27A). This may support the previously reported idea that AVGs are different from SGs [17, 18]. However the relationship between the larger size of AVGs and its distinctive function from SGs is yet uncertain. Real time investigations on cells expressing both AVGs and SGs at the same time and methods to dissociate either of them may provide further differences between them. A previous report conducted such experiment with cycloheximide to dissociate SGs but not AVGs [18]. Photoswitchable fluorescent proteins tagging RIG-I and different components involving antiviral or stress response under such experiment will provide real time super-resolution imaging analysis [26-28].

**Materials and Methods**

**Infection/transfection and stress conditions, and reagents**
Mouse embryonic fibroblasts (MEF) cells with GFP fused MAVS (provided by Zhijian J. Chen (UT Southwestern) or MEF cells from ATTC® (CF-1, ATCC® SCRC-1040) were grown in DMEM supplemented with 10% FBS and 1% PENSTREP at 37°C. IAVΔNS1 is provided by Adolfo Garcia-Sastre (Mount Sinai Hospital). Cells were seeded on Lab-Tek® 8-well chambers, 24 hours prior to infection or transfection. Cells were transiently transfected with 5 µg poly I:C by using JetPrime™ transfection reagent (Polyplus transfection, France), or 1 µg poly I:C by Lipofectamine® 2000 (ThermoFisher Scientific, 11668-019). Cells were infected with IAVΔNS1 in serum-free medium, at MOI:5. After adsorption for 1 hour, virus containing medium is changed to serum-containing medium. For other stress conditions, 0.5 mM sodium arsenite (Sigma) was added to the cell culture for 1 hour. Also for heat shock, cells were incubated at 44 °C, for 30 minutes. For immunofluorescence, RIG-I primary antibody was provided by Takashi Fujita (Kyoto Univ.) and then later purchased (28137, IBL America). TIAR antibody was from Santa Cruz Biotechnology® (sc-1749).

**Immunofluorescence protocol**

MEF cells were fixed by 3.7% paraformaldehyde for 10 minutes in the room temperature, and washed three times by PBS. The fixed sample was permeabilized by 0.5% TritonX for 15 minutes and washed three times by PBS. Permealized samples were then incubated with Image-iT® Signal Enhancer (ThermoFisher Scientific I36933) for 30 minutes in the room temperature, for blocking nonspecific binding of dyes. Then samples were washed three times by PBS, and then incubated with the general blocking buffer (3% BSA+0.05% Tween 20) for one hour at room temperature. Then permeabilized and blocked samples were incubated with the primary antibody (diluted in 0.3% BSA to be the final concentration of 4 µg/ml) for 1.5 hours at the room temperature. Then the samples were washed three times with 0.3% BSA, in five minutes interval, and incubated one hour with 2nd antibody that was pre-labeled with Alexa Fluor® 647 (A647) and Alexa Fluor® 405 (A405) dyes at the room temperature. For optimal blinking of A647 dyes for the super-resolution imaging, per single antibody ~0.7 A647 and ~3 A405 dyes were labeled. Then the samples were washed three times by 0.3% BSA.

**Imaging buffer condition**
Our stock imaging buffer contains 10mM NaCl, 50mM Tris-Cl (pH 8.5) and 10% glucose. Right before imaging, β-mercaptoethanol (M6250, Sigma-Aldrich) was put (1% final concentration) as A647 blinking inducing agent, and catalase (219001, EMD Millipore, 909 unit/ml) and Pyranose Oxidase (P423, Sigma-Aldrich, 4.44 unit/ml final concentration) were put as the oxygen scavenging system. Per each imaging chamber, 500uL of buffer was put before imaging.

**Instrumentation for 3D super-resolution imaging**

The detail of the setup is described in a previous report [22, 23]. Briefly, we use an inverted optical microscope (Olympus IX-71 with Olympus 100X NA 1.4 SaPo oil immersion objective) with a red laser (647nm, 100mW, Crystalaser, DL640-100-AL-O) and a violet laser (405nm, 10mW, SpectraPhysics, Excelsor). The lasers are combined through dichroic mirrors, going through a beam expander with 7.5 expansion. Then the expanded and collimated beams are guided to go through a TIR lens which focuses the beams at the back focal plane in the microscope with an angle slightly smaller than total internal reflection (TIR) angle, to have reduced background but still illuminate several hundred nm in z. All the laser lines were reflected by a dichroic mirror (Semrock R405/488/561/635-Di01-25X36, or Semrock FF408/504/581/667/762-Di01-25X36) to the objective. The emission signals was collected by objective, passed through an emission filter (Semrock FF01-594/730-25) and two additional notch filters (Semrock NF01-568/647-25X5.0 and NF01-568U-25) to clean up the excitation laser, and imaged on a 512x512 Andor EMCCD camera (DV887ECS-BV, Andor Tech). For 3D imaging, a cylindrical lens with a focal length of 2 m (SCX-50.8-1000.0-UV-SLMF-520-820, CVI Melles Griot) was inserted in the emission path. For each imaging of one super-resolution imaging, 30,000 frames of images were taken with 30ms exposure.

Since the super-resolution imaging takes 15 minutes for the same imaging area, it is essential to ensure horizontal and vertical stability against drift. The horizontal drift is corrected later by data analysis, and the z-drift is fixed by pairing PI piezo-objective (P-721.10) and ASI CRIFF. The controllers of them are connected to communicate with each other to ensure establishing a feedback loop. Image acquisition was controlled by a home written data acquisition program (C++).

**Data analysis software**
The data analysis algorithm is provided by Xiowei Zhuang (Harvard Univ.) [22] and was modified for 3D imaging [23].

**Peak Identification and Fitting**

In order to get rid of noises from various sources such as the EMCCD sensor or ambient lights, images are first averaged through blurring by Gaussian convolution of 9X9 pixels. To get the average fluctuation of the signal and noise in individual frames, the standard deviation of the intensity in each frame is calculated. All the pixels with intensity values greater than the 3.5-4.5 fold of the standard deviation in each frame are identified. Within 5-by-5 pixel area, local maximum intensity pixels whose intensity values are greater than its 24 surrounding pixels are found to represent the intensity peak of a single fluorophore. To avoid overlapping fluorophores, two additional filters are applied based on the sharpness and roundness of the identified peak. Sharpness is defined as the intensity ratio between the peak and the background. Roundness is defined as the ratio between x variance and y variance in 9X9 pixels. For identified peaks that passed the sharpness and roundness filters, a square region of 19X19 pixels surrounding local maximum intensity pixel is fitted with an Elliptical Gaussian function,

\[
G(x,y) = h \exp \left( -2 \frac{(x-x_0)^2}{w_x^2} - 2 \frac{(y-y_0)^2}{w_y^2} \right) + b
\]

, where b is the background level, h is the amplitude of the peak, \(w_x\) and \(w_y\) are elliptical widths, \(x_0\) and \(y_0\) are the center coordinates of the peak. If the software fails to fit any 19X19 square region of pixels with a Gaussian function, then that region is discarded. The z-positions of the fluorophores are determined by comparing their \(w_x\) and \(w_y\) values to a calibration curve. The calibration curve is generated from distributions of \(w_x\) and \(w_y\) values from image of fluorescently labeled single antibodies as objective is moved by defined distance along z direction. Then to avoid double counting the same fluorophore, the software connects molecules appearing in consecutive frames by generating trails. Then further filters are applied on each peak in every trail, to remove unqualified peaks. The final localization of the molecule is calculated from the average of center positions of the peaks within the trail, weighted by photon numbers in each frame.

**Cluster analysis**

For cluster analysis of our super-resolution images, a density based clustering analysis algorithm, DBSCAN [29] was used, as previously reported [30, 31]. Briefly, points in super-resolution image are grouped into clusters based on their spatial density. With two required parameters, Npts and Eps, clusters are starting to form from high density spots termed core points. All the core points of a starting cluster are within Eps distance with each other, and they are surrounded by at least Npts number of points. Those clusters can grow by incorporating border points which are the points
located within Eps distance to any of the core points, but are not to each other. Eps=37nm and Npt=7 are empirically chosen (Supplementary data). The analysis gives various data including 1) total number of clusters; 2) the average size of clusters. Here the “size” of a cluster is defined as the twice of the average distance from the center of the cluster to every point in the cluster, so it can be considered as the average diameter of a cluster by fitting to a sphere.

**Area of AVGs/SGs calculation**

The areas of AVGs/SGs were calculated by NIH ImageJ. The original super-resolution images were converted into 8-bit, then the noise was removed by RemoveOutliners function. Then they were converted to be binary images, and areas of AVGs/SGs were recognized by Analyze Particle function.
Figures and Tables

Figure 21. Antiviral signaling pathway against dsRNA virus

(A) Upon the invasion of a double stranded RNA (dsRNA) virus, retinoic acid-inducible gene-I (RIG-I) senses it and starts the cascade of antiviral signaling pathway. RIG-I activates mitochondrial antiviral signaling protein (MAVS). MAVS then activates transcription factors to induce interferon (IFN) which activates PKR that blocks eukaryotic initiation factor 2 (EIF2) activity. This leads to translation inhibition of viral gene, blocking the replication of virus. IFN also can send antiviral signals to other cells, or cause apoptosis if no other means of defense is available. (B) Some viruses can block this defense mechanism. For example, influenza can encode nonstructural one (NS1) protein that can block the antiviral signaling pathways, so that INF induction is inhibited while influenza can replicate utilizing the translation pathway of the host.
Figure 22. RIG-I localizes to antiviral granules (AVGs) under dsRNA infection/transfection and various stress conditions. (A) MEF cells that are fixed 12 hours post poly I:C transfection, and are immunofluorescently stained for RIG-I and TIAR. (B) MEF cells are infected with IAVΔNS1 for 12 hours, treated with sodium arsenite (NaAsO₂) for 1 hour, or exposed to heat (44 °C) for 30 minutes. Cells were fixed and stained for RIG-I.
Figure 23. RIG-I in an AVG. (A) Conventional image of an AVG in a MEF cell, 12 hours after poly I:C transfection. (B) the corresponding super-resolution image.
Figure 24. RIG-I forms clusters in AVGs. (A) Super-resolution image of RIG-I in a MEF cell displaying several AVGs. (B) A zoomed image RIG-I in of a single AVG (upper panel) and a zoomed image of TIAR in a single AVG (lower panel). (C) Clusters of RIG-I (upper panel) and TIAR (lower panel) identified by DBSCAN cluster analysis algorithm are plotted in various colors.
Figure 25. RIG-I in intrinsically clustered but after poly I:C transfection the size of clusters became smaller in cytoplasm and bigger in AVGs (A) The percentage of RIG-I localizations in clusters in MEF cells upon poly I:C transfection and mock-treatment. 37 AVGs were analyzed from six cells under poly I:C, and 30 areas of cytoplasm were analyzed from the same cells. For the mock case 23 areas from three uninfected cells were analyzed. (B) The average size of RIG-I clusters in MEF cells upon poly I:C transfection and Mock-treatment. (C) Super-resolution RIG-I images in MEF cells with poly I:C transfection or mock-treatment.
Figure 26. RIG-I forms clusters in AVGs. (A) The percentage of localizations in RIG-I, TIAR, and 2nd antibody clusters in MEF cells upon poly I:C transfection. For RIG-I, 37 AVGs and 30 cytoplasm areas were analyzed from six cells under poly I:C transfection. For TIAR, 19 AVGs and 20 cytoplasm areas were analyzed from five cells under poly I:C. For the 2nd antibody control case, 11 areas were analyzed from three cells which underwent every step of immunofluorescence except the primary antibody. (B) The average size of RIG-I, TIAR, and 2nd antibody clusters in MEF cells upon poly I:C transfection. (C) The percentage of localizations and the average size of clusters are plotted together for five different cases. Each dot represents either an AVG or an area. For (A), (B), and (C), the numbers plotted for the 2nd antibodies are from two 2nd antibodies against RIG-I primary antibody and TIAR primary antibody respectively (Table 4).
Figure 27. RIG-I forms clusters universally. (A) Super-resolution images of RIG-I in granules in MEF cells 12 hours after IAVΔNS1 infection, 1 hour after sodium arsenite (NaAsO₂) treatment or 30 minutes after heat shock (44 °C). (B) Percentage of RIG-I localizations in clusters in granules for three cases (left panel) and the average size of RIG-I clusters in granules for the three cases (right panel). 18 AVGs from three cells under IAVΔNS1, 57 SGs from nine cells under NaAsO₂, and 18 SGs from four cells under heat shock were analyzed. (C) The percentage of localizations and the average size of clusters are plotted together for the three cases with the 2nd antibody as a control.
Figure 28. AVGs are bigger than SGs. (A) The average size of AVGs or SGs upon dsRNA infection/transfection, sodium arsenite (NaAsO₂) treatment (1 hour), and heat shock (44 °C, 30 minutes). 96 RIG-I AVGs and 28 TIAR AVGs from poly I:C and IAVΔNS1 transfection/infection, 186 RIG-I SGs and 70 TIAR SGs from NaAsO₂, and 36 RIG-I SGs and 40 TIAR SGs from heat shock were analyzed. (B) Examples of super-resolution images of RIG-I and TIAR for three cases.
### Percentage of clustering and sizes of clusters upon different conditions

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<td>NaAsO&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>granu.</td>
<td>58(±1)%, 112(±2)nm, (37 AVGs, 6 cells)</td>
<td>58(±2)%, 107(±3)nm, (18 AVGs, 3 cells)</td>
<td>53(±1)%, 106(±2)nm, (57 SGs, 9 cells)</td>
<td>56(±3)%, 86(±2)nm, (18 SGs, 4 cells)</td>
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<td>58(±2)%, 77(±1)nm, (30 areas, 6 cells)</td>
<td>45(±5)%, 73(±3)nm, (3 cells)</td>
<td>42(±8)%, 75(±4)nm, (9 cells)</td>
<td>52(±5)%, 69(±2)nm, (4 cells)</td>
<td>48(±2)%, 93(±2)nm, (23 areas, 3 cells)</td>
</tr>
<tr>
<td>TIAR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>granu.</td>
<td>15(±3)%, 85(±2)nm, (19 AVGs, 5 cells)</td>
<td>27(±3)%, 112(±5)nm, (8 AVGs, 2 cells)</td>
<td>26(±3)%, 115(±6)nm, (7 cells)</td>
<td>25(±4)%, 91(±6)nm, (4 cells)</td>
<td>N/A</td>
</tr>
<tr>
<td>cyto.</td>
<td>11(±2)%, 69(±2)nm, (20 areas, 5 cells)</td>
<td>23(±2)%, 75(±4)nm, (10 areas, 2 cells)</td>
<td>18(±5)%, 86(±3)nm, (7 cells)</td>
<td>16(±3)%, 68(±2)nm, (4 cells)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Control w/o primary antibody (secondary antibody only)

secondary antibody against RIG-I primary antibody: 24(±1)%, 65(±1)nm, (6 areas, 2 cells)

secondary antibody against TIAR primary antibody: 25(±3)%, 58(±2)nm, (5 areas, 1 cell)

RIG-I in AVG by poly I:C, by a different secondary antibody: 69(±2)%, 126(±4)nm, (28 areas, 6 cells)

Table 4. Percentage of clustering and sizes of clusters upon different conditions

Each entry in the table shows “the percentage of clustering,” “the average size of clusters,” “the number of AVGs/SGs/areas for the analysis,” “the number of cells from where AVGs/SGs/areas came from”.

The percentage of clustering is defined by the percentage of points included in the detected clusters in an area of interest, by the cluster analysis algorithm. The size of a cluster is defined as the twice of the average distance from the center of the cluster to every point in the cluster, so it can be considered as the “diameter” of the cluster. Mock treated case is the first control (the last column). Two other controls that don’t fit in the main table are put under it.
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Chapter 3

The morphology of RecA bundles upon the SOS response

Introduction

In this chapter we will investigate the bacterial reaction to the vast global DNA damage, using our super-resolved single-fluorophore microscopy technique. The SOS response is an inducible DNA repair system that enables bacterial cells to survive under sudden increases in DNA damage. Many antibiotics are known to cause it, and it eventually leads to the emergence of mutant strains resistant to previous antibiotics, causing medical alerts [1].

Antibiotics can be classified in five different categories by the mechanisms by they operate [2]. Antibiotics like β-lactams interfere with cell wall synthesis. Antibiotics like daptomycin disrupt membrane structure. Antibiotics like aminoglycosides or chloramphenicol inhibit protein synthesis. Antibiotics like trimethoprim or sulfonamides inhibit metabolic pathway. Antibiotics like fluoroquinolones interfere with nucleic acid synthesis (Fig. 29). It has been reported that out of 34 antibiotics, 23 (68%) of them are causing SOS response. Thus SOS does matter in the clinical application of antibiotics [1, 3-5].

SOS response is initiated by RecA. RecA is a 38 kiloDalton protein essential for the homologous recombination repair of dsDNA break (DSB) and maintenance of DNA [6]. The role of RecA in the dsDNA break was reported comprehensively by the mean of Structural Illumination Microscopy (SIM) previously [7]. After RecBCD procession of the DSB, RecA is recruited to the cut locus and nucleates into a RecA bundle. This bundle extends along the long axis of the cell and connects the cut locus to the uncut sister locus (the homology site). Then the cut and uncut loci are brought together, the RecA bundle disassembles and the proteins that act later in the homologous recombination are recruited.
In contrast to the single RecA bundle appearing in case of a single DSB, many RecA bundles and foci appear in the SOS response when the DNA is damaged globally. The known order of SOS response is the following. When the DNA is vastly damaged, ssDNA (single stranded DNA) is around as a byproduct and binds to RecA. Then SOS repressor LexA is cleaved by RecA, releasing SOS regulatory proteins including RecA, UmuC, UmuD, SulA, UvrA, UvrB, DinA, DinB, DinF and etc. About 40 genes were shown to be under SOS control. Then this will lead to make more RecA and other SOS related proteins be produced and accelerate the process of the repair (Fig. 30B). Once the repair is done the level of RecA reduces (Fig. 31). The SOS response is an error prone process, introducing errors in the genome. Therefore strains having antibiotics resistance would emerge, as a result of mutation (Fig. 30A) [8].

Though it was reported that in the SOS response RecA relocalizes into foci [6], and the previous study revealed the emergence of RecA bundles in the case of DSB [7], further details of the morphology of RecA bundles in foci are not known well due to the resolution limit of conventional microscopy as well as SIM. Here we report that RecA bundles in SOS responses are forming ribbon-like structures, i.e. flat at one side and wide at the other side, hypothetically wrapping around DNA damaged sites, under our super-resolved single-fluorophore microscopy [9, 10].

To visualize the boundary of bacterial DNA, we used a strain having HU proteins tagged with GFP. Hu is a small, heat-stable DNA binding protein. In *E. coli*, HU is a heterodimer composed of two highly homologous subunits of 9 kiloDalton each. HU contributes to the maintenance of DNA superhelical density and to modulate topoisomerase I activity. HU participates in DNA repair via RecA-dependent pathway, but the precise mechanism is unknown [11]. In this report we imaged HU together with RecA to visualize the DNA boundary together with RecA filaments upon DNA damage.

**Methods**

**Cell preparation and DNA damage treatment**

RecA-GFP *E. coli* (SS-3085) cells were provided by Steven Sandler (Univ. of Massachusetts, Amherst) were culture overnight in LB media. Next day live cells were cultured in LB in 37 °C
until optical density (OD) reaches at 0.4. Then the cell culture was mixed with MMC (Mitomycin C with 0, 5, 10 and 20 µg/ml concentrations) and was continuously incubated. At each time point within 10~300 minutes, a drop of cell was sandwiched between a coverslip and agarose gel, and was imaged under Nikon Ti-2000 microscope (Fig. 31). HU-GFP *E.coli* cells (LMC500/PBAD-hupA-gfp) provided by Josette Rouviere-Yaniv (Laboratoire de Physiologie Bactérienne) were cultured overnight in LB, next day cultured in LB with MMC (Mitomycin C, 5µg/ml). MMC is a DNA crosslinking agent that leads to DNA damaging. At different time points, cells were fixed and put on the chambers for immunofluorescence. RecA antibodies (Abcam ab63797 or B-Bridge 61-003) and HU antibody (Rockland 600-106-215) were used. RecA antibody was “washed” with ΔRecA *E.coli* (SS-5912 from Steven Sandler) cells before use.

**Immunofluorescence protocol**

MMC (5 µg/ml, 5, 10, 15 and 20 minutes post treatment) treated HU-GFP cells were centrifuged (1000g, 3 minutes in 4ºC), and the pallet was mixed with 3.7% paraformaldehyde. At the same time, cells were put on poly-L-lysine coated Lab-Tek® 8-well chambered coverglass, so fixation and immobilization were done at the same time for 30 minutes at room temperature, and washed three times by PBS. The fixed sample was permeabilized by 1mg/ml lysozyme for 30 minutes and washed three times by PBS, being followed by blocking by 3% BSA for one hour at room temperature, or at 4ºC for longer storage. In the meantime, the primary antibody for RecA was “washed,” that is, additionally purified for eliminating the nonspecific binding of it. The washing protocol is the following.

ΔRecA (RecA deletion strain) cells were incubated overnight in LB, and the next day incubated in LB again to reach OD=0.6. 3mL of ΔRecA cell culture is sufficient for washing 1µg of RecA primary antibody that is sufficient for staining up to 8 samples. ΔRecA culture was centrifuged (4000g, 5 minutes in 4ºC) and mixed with 3.7% paraformaldehyde and incubated for 30 minutes in 1.5ml Eppendorf® tubes at room temperature. Then fixed cells were washed two times by PBS by centrifugation (4000g, 3 minutes), and permeabilized by 1mg/ml lysozyme for 30 minutes room temperature. Permeabilized cells were washed twice by PBS by centrifugation (1000g, 5 minutes), then all the pallets were combined together and mixed with RecA primary
antibody in 1/1000 dilution (1µl in 1000µl PBS) in a 1.5ml Eppendorf® tube. RecA antibody was incubated with permeabilized ΔRecA cells for one hour in a Labquake® shaker at the room temperature. Then the tube was centrifuged (1000g, 5min) and the supernatant was moved to another 1.5ml Eppendorf® tube and centrifuged once again (20000g, 1min) to remove all the ΔRecA cells and their debris. The supernatant was moved to another tube and mixed with 100µl 3% BSA to give antibody solution in 0.3% BSA.

With this RecA primary antibody, permeabilized and BSA blocked HU-GFP cells were incubated for 1~1.5 hours at the room temperature. Then the samples were washed three times with 0.3% BSA, in five minutes interval, and incubated one hour with 2nd antibody that was pre-labeled with Alexa Fluor® 647 (A647) and Alexa Fluor® 405 (A405) dyes at the room temperature. For optimal blinking of A647 dyes, per single antibody ~0.7 A647 and ~3 A405 dyes were labeled. Then the samples were washed three times by 0.3% BSA.

For imaging HU, anti-GFP antibody was mixed with “washed” Rec-A primary antibody and put on the samples and incubated together. For the 2nd antibody step, 2nd antibody labeled with Alexa Fluor® 568 (A568) and A405 targeting anti-GFP antibody was mixed with the 2nd antibody targeting RecA primary antibody, and put on the samples and incubated together. However A568 does not give a detailed super-resolution image as A647 does, so the labeling was switched when detailed HU images were needed.

**Imaging buffer condition**

Our stock imaging buffer contains 10mM NaCl, 50mM Tris-Cl (pH 8.5) and 10% glucose. Right before imaging, β-mercaptoethanol (M6250, Sigma-Aldrich) was put (1% final concentration) as A647 blinking inducing agent, and catalase (219001, EMD Millipore, 909 unit/ml) and Pyranose Oxidase (P423, Sigma-Aldrich, 4.44 unit/ml final concentration) were put as the oxygen scavenging system. Per each imaging chamber, 500uL of buffer was put before imaging.

**The super-resolution microscopy**

The details of the 3D super-resolution microscopy was described previously [10]. Briefly, Olympus IX-71 inverted microscope with 100X NA 1.4 SaPo oil objective was used with red (DL640-100-AL-O, Crystalaser) and violet (405nm, 20mW, SpectraPhysics Excelsor) lasers.
Mechanical shutters (LS6T2, Unibliz) were used to alternatively excite the sample with the two lasers. The lasers were expanded by 7.5X, reflected by a dichroic mirror (Semrock Di01-R405/488/561/635), and sent to the sample chamber with a focusing lens that also creates an incidental angle slightly smaller than the total internal reflection angle. This reduces the background signal. The emission was collected by the objective, passing through an emission filter (Semrock FF01-594/730-25 for A647 or Chroma HQ585/70M 63061 for A568) and two additional notch filters (Semrock NF01-568/647-25X5.0 and NF01-568U-25), and was imaged on an EMCCD camera (DV887ECS-BV, Andor Tech). A cylindrical lens (SCX-50.8-1000.0-UV-SLMF-520-820, CVI Melles Griot, 2m focal length) was in the emission beam path to induce astigmatism for 3D detection. To ensure z-directional stability in the data acquisition, ASI CRIFF (Applied Scientific Instrumentation) with a piezo-objective (PI P-721.10) were used to compensate the vertical drift. The horizontal drift was corrected in the post data acquisition step, by the analysis software utilizing the correlation function. The data analysis software was provided by Xiaowei Zhuang (Harvard Univ.) and modified for 3D imaging.

**Cluster analysis**

A density based clustering analysis algorithm, DBSCAN was used [12], for cluster analysis of our super-resolution images as previously reported [10]. Briefly, spots in super-resolution image are grouped into clusters based on their spatial density. With two required parameters, Npts and Eps, clusters are starting to form from high density spots termed core points. All the core points of a starting cluster are within Eps distance with each other, and they are surrounded by at least Npts. Then such a cluster can grow by incorporating border points which are the points located within Eps distance to any of the core points, but are not to each other. Eps=38nm and Npt=10 are empirically chosen. The analysis gives various data including 1) total number of clusters; 2) the average size of clusters. Here the “size” of a cluster is defined as the twice of the average distance from the center of the cluster to every point in the cluster, so it can be considered as the average diameter of a cluster by fitting to a sphere.

**Skeletonizing and Disks fitting analysis**

A localized version of $L_1$-medial skeleton of 3D point cloud analysis [13] was done on each individually super-resolution images of *E.coli* cell to create curve skeleton structures along RecA
bundles. This algorithm does not require the input shape to be complete, watertight or represented by fine tessellation. The input can be also unorganized and unoriented with significant noise and outliers. The analysis starts from randomly down-sampled samples from the input points. The initial down-sampling number is one of the major input parameters in this algorithm. Each sample point is iteratively projected and redistributed to the center of the input points within its local neighborhood. The neighborhood size is another major input parameter. Then this neighborhood is gradually growing to handle structures of different levels of details. The neighborhood growth rate is another major input parameter. In this process, the branches are constructed, expanded and connected. Then down-sampling, smoothing and re-centering are applied to enhance the result, and it finally generates sets of well-connected curve skeleton points. The skeletons represent one-dimensional local centers of the input shape. Applying the L1-median alone tends to generate artificial clustering of local centers. To avoid it, a repulsion force is included in the algorithm to be applied whenever a skeleton branch is formed locally. This repulsion force is the final major input parameter.

Then to determine whether RecA bundles are pipe-like (circular at the intersection) or ribbon-like (flat at the intersection), ellipses are being fit to the original data points, in planes perpendicular to the skeletons (Fig. 35, 36, the third and fourth row images). Thus the center of the ellipses are not necessarily on the skeletons. This analysis software was designed and provided by Dr. Huy Bui (Van Gogh Imaging).

**Results**

**RecA increases in expression level and forms foci under DNA damage while Hu remains same**

E.coli cells are fixed at different time points (5, 15, and 20 minutes) after the incubation with MMC. RecA and Hu are immunofluorescently labeled and imaged under an optical microscope (Nikon TE2000). RecA level rapidly jumps up by several folds in 5~15 minutes than the control (without MMC) case. Also RecA redistributes on foci, and RecA in background reduces significantly in the meantime. However from 15~20minute time points, RecA distribution starts to become uniform (Fig. 32, upper panel). This result is comparable to the trend of RecA-GFP
upon DNA damage (Fig. 31), but the SOS response time is much faster than RecA-GFP. In contrast, Hu level and distribution remained the same (Fig. 32, lower panel).

RecA distribution in intact cells and DNA damaged cells show distinct characteristics

Among intact cells, uniform distribution of RecA distribution is observed by super-resolution imaging. The distribution of RecA is within the boundary of in HU distribution, so RecA distribution was low in the cytoskeleton, assuming HU also resides in the E.coli chromosome. (Fig. 33A, B). On the other hand, cells with DNA damage displayed discontinuous helical bundles structures (Fig. 33C, D, E). Compared to 10 minutes, in 15 minutes RecA distribution on foci became less dense and there are more RecA elsewhere, even though RecA bundles still keep the helical shape (Fig. 33D, E). A 568 dye does not provide as complete image as A647 dye does, so the dotty distribution or polar distribution of Hu are artificial. Thus we switched the labeling and obtained more uniform Hu distribution (Fig. 34), consistent with earlier report. RecA distribution in this case also displays discontinuous helical bundles. In all the cases RecA distribution stayed in the boundary of HU distribution, so conclude that RecA mostly stays very close to E.coli chromosome.

RecA bundles are ribbon like, instead of pipe-like

To determine the morphology of RecA bundles upon DNA damage, we applied a localized version of L1-mediated skeleton algorithm as well as disks fitting to the RecA images of HU-GFP cells. However, applying skeletonizing algorithm directly to the original raw data of MMC treated cells caused the rise of excessive small branches and overlapping disks (Fig. 35A, C, the third, fourth and the fifth row images). Thus we tried applying DBSCAN cluster analysis first to the raw data, then applied skeletonizing and disks fitting analysis to them, and this yielded much cleaner skeletons generation as well as disks fitted (Fig. 35B, D, the third, fourth and fifth row images).

On the other hand, conducting cluster analysis on RecA images of intact cells was not favorable, since RecA is generally uniform in these cells so that doing so left too few points (Fig. 36B, the first and row images). This leads to fragmented skeletons with overlapping disks (Fig. 36B, the third and fourth row images) after skeletonizing and disks fitting analysis. In contrast, the
original raw data gives a clean skeleton along the cell axis and disks along to it (Fig. 36A, the third and fourth row images).

Therefore the best analysis for the RecA images in that cluster analysis followed by skeletonizing and disks fitting for MMC treated cells, and skeleton analysis and disks fitting only for intact cells. This combination gives the highest contrast in plotting the axes ratio, i.e. (longer axis)/(shorter axis) distributions in different conditions of MMC treatment (Fig. 37C), while other combinations (without cluster analysis for all, or with cluster analysis for all) give less contrast in the axes ratio distributions (Fig. 37A, B). The average axes ratio for the intact cells (no MMC) is 1.24, while the average axes ratios for the MMC treated cells are 1.64, 1.53 and 1.64 respectively to 5µg/ml, 10µg/ml and 15µg/ml MMC concentrations. Therefore we conclude that RecA bundles under DNA damage are “ribbon-like,” instead of “pipe-like.”
Antibiotics can be categorized by five different mechanisms by which they interfere or inhibit the viability of bacterial cells.

**Figure 29.** Mechanisms of how antibiotics kill bacterial cells [2]

Antibiotics can be categorized by five different mechanisms by which they interfere or inhibit the viability of bacterial cells.
Figure 30. RecA is an essential player in the SOS response

(A) When the DNA is globally damaged in bacterial cells, RecA aggregates around the damaged sites and form foci. The SOS response is an error prone response, leading to mutation of cells, contributing to the emergence of antibiotics resistant strains. (B) The order of SOS response. As byproduct of DNA damage, ssDNA binds to RecA and this "activated" RecA cleaves LexA. This releases SOS regulatory proteins repressed by LexA and RecA level goes up.
Figure 3. RecA-GFP level goes up by DNA damage, then eventually goes down.

(A) Live RecA-GFP cells are incubated in the presence of MMC in different concentrations (0, 5, 10, 20 µg/ml), and were imaged in different time points. (B) The average intensity per pixel in each cell is defined as the sum of all pixel values of a cell, then divided by the pixel area of it. Each point represents average of around 30 cells.
Figure 32. HU-GFP cells. RecA level increases over the time after MMC was injected in the cell culture, but HU level remains the same.
**Figure 33.** RecA distributions in intact cells or DNA damaged cells show distinct characteristics

(A) An intact cell rotated around its long axis by 45° in each step. (B) Five examples of intact cells. (C) A single cell with DNA damage (10 minutes after MMC) showing rotational image by 45° in each step. (D) Three examples of cells 10 minutes after MMC treatment. (E) Three examples of cells 15 minutes after MMC treatment (Color convention: **Red** for RecA-A647, **Green** for HU-A568).

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**Figure 34.** HU is uniformly distributed

A single cell with DNA damage (10 minutes after MMC) showing rotational image by 45° in each step. HU shows uniform distribution, while RecA bundles wrap around HU distribution (Color convention: Red for HU-A647, Green for RecA-A568).
Figure 35. Skeletonizing and disks fitting to RecA bundles of individual MMC treated cells

(A) Original coordinates of the final localizations of a single cell fixed at 10 minutes after MMC treatment. The coordinates are visualized as a projection on the XY plane (two dimensional projection), three dimensional stereoscopic representation, and skeletons with fitting disks, a rotated view of the previous, and a zoomed image of a selected area in the previous. One disk is colored red for better view of the representation. (B) The same cell went through DBSCAN cluster analysis, and the clusters are visualized in the same order as previously. (C)(D) A single cell representations as previously, but for a different cell fixed at 15 minutes after MMC treatment.
Figure 36. Skeletonizing and disks fitting to RecA bundles of an individual intact cell

(A) Original coordinates of the final localizations of an intact fixed cell. The coordinates are visualized as a projection on the XY plane (two dimensional projection), three dimensional stereoscopic representation, and skeletons with fitting disks, a rotated view of the previous, and a zoomed image of a selected area in the previous. One disk is colored red for better view of the representation. (B) The same cell went through DBSCAN cluster analysis, and the clusters are visualized in the same order as previously.
Figure 37. Three different ways of plotting the axes ratio for different MMC treatments

(A) The distribution of axes ratio, i.e., (longer axis)/(shorter axis) of the fitting disks to the original coordinates of individual cells. 

(B) The distribution of fitting disks axes ratio of the coordinates of individual cells after cluster analysis. 

(C) The distribution of the fitting disks axes ratio of the original coordinates of individual intact cells, and MMC treated individual cells after cluster analysis.
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Chapter 4

Plantazolicin is an ultra-narrow spectrum antibiotic that targets the B. anthracis membrane

In this session we will discuss the application of our super-resolution imaging capability to another example of fighting bacterial cells’ resistance to antibiotics. The conventional approach of applying broad-spectrum antibiotics to treat bacterial infections contributes to the emerging of antibiotic resistance. To cope with this, species specific and narrow-spectrum antibiotics draw attention as the alternative approach [1, 2]. Also natural sources have been historically valued for their less likeliness of causing side effects [3].

Introduction & results

Activity spectrum of PZN

Plantazolicin (PZN) is a natural antibiotic produced from Bacillus amyloliquefaciens FZB42 and Bacillus pumilus. It is a polyheterocyclic, linear compound of the ribosomally synthesized and post-translationally modified peptide (RiPP) natural product family [4]. The canonical producing strain, B. amyloliquefaciens FZB42, has been known to have antifungal and nematicidal activity and it’s a prolific producer of other natural products. Even though one previous study speculated PZN has nematicidal activity since PZN-deficient FZB42 strains exhibited reduced activity against Caenorhabditis elegans, PZN was found to no more toxic to C. elegans than a vehicle control and is not nematicidal in its own right. Purified PZN was also not responsible for the antifungal activity of the native producer, leaving the ecological function of PZN unknown.

However PZN gains its antibiotic property once twelve of the fourteen amino acid residues are tailored. Previously PZN has been reported to have antibiotic activity against Gram-positive
bacterial organisms like *B. subtilis, B. cereus, and B. megaterium* [4]. However this conclusion was reached by an exorbitant amount of purified PZN (1mg per spot).

Using a microbroth dilution assay, the activity of PZN was later found to be far more selective only toward *B. anthracis*, by screening panels of human pathogens [5, 6]. PZN was found to be selectively effective for vegetative *B. anthracis* as well as fully virulent biosafety level 3 strains, with minimal inhibitory concentrations (MICs) of 1~16 µg/ml. PZN is not effective to endospores, the dormant phase of *B. anthracis* life cycle, but becomes effective once germination was initiated. In contrast, *B. subtilis* and *B. cereus* were not susceptible to PZN at up to 64 µg/ml concentration, which is clearly contrary to the previous conclusion.

There have been numerous efforts to elucidate the mode of action (MOA) of PZN [7]. Popular strategies include transcriptional profiling of the gene expression of an affected organism [8], identification of genomic polymorphisms among resistant mutants of a susceptible strain [9], inhibition of macromolecular biosynthesis [10], affinity purification of the molecular target [11], and under or overexpression of the putative target [12]. However these methods meet challenges if the compound of interest acts upon a multicomponent cellular structure like bacterial membrane.

*B. anthracis* is a Gram-positive bacterium and it belongs to *B. cereus sensu lato* group, which includes *B. cereus, B. anthracis, B. thuringiensis, and B. mycoides* [13, 14]. Despite being grouped with other *Bacillus* species, *B. anthracis* has several characteristics that make it distinct from other members of the *B. cereus* group. It is an agent of anthrax and a category A priority pathogen. A fully virulent *B. anthracis* contains two conservative plasmids, pXO1 and pXO2, encoding the genes responsible for producing the anthrax toxin and poly-D-glutamic acid capsule. Other than this characteristic plasmid content, unlike other members of the *B. cereus* group, *B. anthracis* harbors a nonsense mutation in *plcR* (phospholipase C regulator) which yields a truncated and inactive protein. Thus *B. anthracis* does not have phospholipase C activity [15].

*B. anthracis* has a two-dimensional protein lattice that encompasses the entire cell, exterior to the cell wall. This layer is composed by the surface layer (S-layer) and a 2nd cell wall polysaccharide (SCWP). The S-layer is non-covalently attached to the cell by lectin-like interactions with SCWP.
The S-layer is decorated with surface association proteins [18]. The B. anthracis SCWP is species specific [19, 20] and a binding site of gamma (γ) phage [21, 22].

γ phage produce a peptidoglycan hydrolase, PlyG, which specifically recognizes the terminal galactoses of B. anthracis SCWP and subsequently hydrolyze the cell wall [21]. However other major members in B. cereus sensu lato group have terminal glucose moieties in their SCWP and so resistant to γ phage lysis. However there exist atypical B. anthracis strains which lack the galactose-forming UDP-glucose 4-epimerase. Such strains are resistant to γ phage lysis and would constitute false-negative in any diagnostic assay based on γ phage [23]. On the other hand, there exist atypical strains of B. cereus that encode the galatose-forming UDP-glucose 4-epimerase. These strains are susceptible to γ phage lysis, and constitute to a false-positive in any diagnostic assay based on γ phage [24]. Thus such an assay suffers from the selectivity issues [25]. However since γ phage lysis is dependent on the unique structure of B. anthracis wide type (WT), it is a reasonable speculation that the MOA of PZN is related to the mechanism of γ phage lysis.

A head-to-head comparison between the selectivity of PZN and γ phage lysis was conducted. Prior to modern genomic methods, γ phage sensitivity and other phenotype testing were popular methods for identifying B. anthracis [23]. Even though the method was reported to have 96% positive accuracy, the γ phage lysis assay has been documented to contain a few false positives and negatives [23, 24, 26]. To see if the MOA of PZN follows the same trend as γ phage assay, a panel of atypical B. cereus stains was treated with PZN, and they turned out to be not susceptible to PZN [26-28]. This rules out the possibility that PZN activity follows similar mechanism of γ phage lysis.

In an attempt to define the species specificity of PZN, various bacterial strains without key differences between B. anthracis and B. cereus were tested. Since plcR, encoding the phospholipase C regulator, is nonfunctional in B. anthracis but is intact in B. cereus [15], eliminating plcR activity in B. cereus could be a possible approach to make it act like B. anthracis. However even so did not increase its susceptibility to PZN. Additionally, sortase-deficient strains of B. anthracis, which lack the ability to anchor various proteins to the cell wall, remain susceptible to PZN [29]. Similarly, PZN activity was not dependent on the presence of B. anthracis S-layer, as stains deficient in S-layer assembly or decoration are inhibited by PZN.
Therefore, these data not only highlight the species specific activity of PZN against \textit{B. anthracis}, but also rule out the S-layer and the SCWP as targets of PZN.

**Assessing potential macromolecules as the target of PZN**

Affinity purification has been a classical technique used to identify targets of bioactive small molecules [11]. In an attempt to isolate the molecular targets of PZN, PZN probes were biotinylated and tested for affinity-based target identification. However no potential binding partners were identified by either mass spectrometry or western blot. Then for the possibility that PZN interacts with a non-protein macromolecules, radiolabelled precursors of the cell wall, fatty acids, and RNA were used to assay for their biosynthesis in the presence of PZN. It turned out PZN disrupted macromolecular biosynthesis [30], but it did not affect cell wall biosynthesis compared to the vancomycin control.

**The gene expression profile of PZN**

Sub-inhibitory antibiotic treatment simulated rapid transcriptional responses in bacteria [31] and the induced/repressed genes may be indicate of MOA [32]. RNA-Seq was performed to evaluate the transcriptional responses to PZN. In an effort to record gene expression changes most relevant to PZN, total RNA was sequenced after treating \textit{B. anthracis} with a sub-lethal (0.25 X MIC) concentration of PZN for a short exposure time (10min) [8]. Totally 74 genes were differentially regulated, and their expressions were validated by qRT-PCR. Fourteen of the upregulated genes were transporter subunits, and this is a common stress response to antibiotic treatment [33]. On the other hand, downregulated genes were associated with L-lactate metabolism for which the implication remained unclear. The most highly upregulated \textit{B. anthracis} genes are homologous to the \textit{B. subtilis} genes \textit{lial} and \textit{liaH}, which are involved in the cell envelope stress response to the antibiotics treatment such as daptomycin [34]. PZN treatment also results in massive upregulation of genes that are homologous to a \textit{B. subtilis} themosensor two-component system that regulates the lipid desaturase [35].

**PZN depolarizes the \textit{B. anthracis} membrane**

The activity of PZN toward \textit{B. anthracis} was examined in the presence of the membrane disrupting agents, nisin and daptomycin. Based on the resulting isobolagrams, both compounds
elicited strong synergistic activity with PZN [36]. Both nisin and daptomycin disrupt membrane potential in Gram-positive organisms, and their synergism with PZN suggests that PZN might also depolarize the *B. anthracis* membrane [37, 38]. Thus DiOC₃(3) (3,3’-diethyloxacarbocyanine iodide) was used to measure the membrane potential of PZN-treated *B. anthracis* cells, and it showed PZN treatment (at both inhibitory and subinhibitory concentrations), correlated with a decrease in membrane potential. This suggests the activity of PZN is related to disrupting the integrity of the cell membrane.

**Localization of PZN**

Since it was evident that PZN targets the cell membrane, we aimed to determine the subcellular localization of PZN by several microscopy techniques. Antibiotics labeled with fluorescent probes have been used previously [39, 40]. Localization of PZN was established by employing a Cy5-labeled PZN derivate (PZN-Cy5) that retains much of its anti-*B. anthracis* activity (MIC of 4 µg/mL). PZN-Cy5 localized to distinct 100~200 nm wide foci. This distinct pattern of PZN labeling was not seen in a spontaneously mutant strain when an excess of unlabeled PZN was administered first, suggesting that PZN and PZN-Cy5 probe interact with *B. anthracis* in an identical fashion.

To study further details of PZN localizations and calculate its clusters, we adapted our super-resolved single-fluorophore microscopy [41], utilizing the blinking characteristics of Cy5 dyes. Our super-resolution image shows PZN localizations are blank in the middle of cells once they are projected along their long axes (Fig. 38 A&B). A probability density plot along the radial direction demonstrates this feature too (Fig. 38C). The PZN clusters can be visually identified that they are around the cell boundary, indicating PZNs are likely to form clusters on the cell membrane (Fig. 39A&B). To determine their sizes, a density based clustering algorithm (more details in the method) [42] was implemented. We obtained the average size of PZN clusters as 181nm (±7nm), and the average number of clusters per cell was 16(±2).

**Methods**

**The super-resolution microscopy**
The details of the 3D super-resolution microscopy was described previously [43-45]. Briefly, Olympus IX-71 inverted microscope with 100X NA 1.4 SaPo oil objective was used with red (DL640-100-AL-O, Crystalaser) and violet (405nm, 20mW, SpectraPhysics Excelsor) lasers. The violet laser served as reactivation laser for the Cy5 dyes. Mechanical shutters (LS6T2, Unibliz) were used to alternatively excite the sample with the two lasers. The lasers were expanded by 7.5X, reflected by a dichroic mirror (Semrock FF408/504/581/667/762-Di01-25X36), and sent to the sample chamber with a focusing lens that also creates an incidental angle slightly smaller than the total internal reflection angle. This reduces the background signal while allowing the illumination of several hundreds nm along z-axis. The emission signal from the sample was collected by the objective, passing through an emission filter (Semrock FF01-594/730-25) and two additional notch filters (Semrock NF01-568/647-25X5.0 and NF01-568U-25), and was imaged on an EMCCD camera (DV887ECS-BV, Andor Tech). A cylindrical lens (SCX-50.8-1000.0-UV-SLMF-520-820, CVI Melles Griot, 2m focal length) was in the emission beam path to induce astigmatism for 3D detection [41]. To ensure z-directional stability in the data acquisition, ASI CRISP (Applied Scientific Instrumentation) with a piezo-objective (PI P-721.10) were used to compensate the vertical drift. The horizontal drift was corrected in the post data acquisition step, by the analysis software utilizing the correlation function [46]. The data analysis software was provided by Xiaowei Zhuang [43] and modified for 3D imaging.

**Immobilization of cells**

Nunc® Lab-Tek® 8-well chambered coverglass (Z734853, Sigma-Aldrich) was coated by 0.1% Poly-L-Lysine (P8920, Sigma-Aldrich) by 30 minutes incubation. Then chambers were dried by nitrogen blowing and cells were incubated for 10 minutes and undetached cells were washed away.

**Imaging buffer condition**

Our stock imaging buffer contains 10mM NaCl, 50mM Tris-Cl (pH 8.5) and 10% glucose. Right before imaging, Cysteamine (M9768, Sigma-Aldrich) was put (10mM final concentration) as Cy5 blinking inducing agent, and catalase (219001, EMD Millipore, 909 unit/ml) and Pyranose Oxidase (P423, Sigma-Aldrich, 4.44 unit/ml final concentration) were put as the oxygen scavenging system. Per each imaging chamber, 500uL of buffer was put before imaging.
Cluster analysis

For cluster analysis of our super-resolution images, a density based clustering analysis algorithm, DBSCAN [42] is used, as previously reported [44, 45]. Briefly, spots in super-resolution image are grouped into clusters based on their spatial density. With two required parameters, Npts and Eps, clusters are starting to form from high density spots termed core points. All the core points of a starting cluster are within Eps distance with each other, and they are surrounded by at least Npts. Then such a cluster can grow by incorporating border points which are the points located within Eps distance to any of the core points, but are not to each other. Eps=40nm and Npt=19 are empirically chosen. The analysis gives various data including 1) total number of clusters; 2) the average size of clusters. Here the “size” of a cluster is defined as the twice of the average distance from the center of the cluster to every point in the cluster, so it can be considered as the average diameter of a cluster by fitting to a sphere. An example of this analysis on three cells is shown (Fig. 40). 14 cells were analyzed in total to give the average size of PZN clusters.

2D projection of cells

To verify if most PZN molecules are localized toward the membrane, we applied 2D projection of multiple cells [Fig. 38B, C]. Cells were aligned along the axis of their length and we set as Y axis. Then the poles and septum of them were chopped to avoid spots near to the Y axis, and they were combined and projected on XZ plane. Also the density of spots per unit pixel of 30X30 nm² was obtained. [Fig. 38B.] The color map is ranging from the densest pixel to the sparsest pixel. Also the radial probability of finding a spot was calculated by counting the number of spots in each radial window of 20nm binning, and normalized by the radial distance R. This probability density was then plotted against the distance from the Y axis [Fig. 38C]. As the color-map and the probability plot suggest, PZN molecules were mostly localized toward to the membrane, and formed highly localized dense structures around.
Figure 38: PZN localizations tend to be hollow around each cell

(A) The 3D image of two cells next to each other is projected on the plane [Top]. Then three segments are selected avoiding cell poles and septum, and zoomed (Middle). Then each segment of these cells is rotated to show the cross-section, revealing the hollow nature of PZN localizations around each cell. (B) Cells were reoriented along their length direction, and projected onto a plane against it. In each pixel of 30X30nm², the total number of spots were counted and plotted by heat map. (C) The probability density of finding a spot at R distance from the length axis of cells was plotted by 20nm binning. (Unit: 10⁻³/nm²)
Figure 39. Super-resolution images of PZN-Cy5 labeled *B. anthracis*.
(A) Two representative cells rotated about the z-axis show distinct non-septal localization of PZN-Cy5. Green, blue, and yellow circles mark three individual foci in each rotated view. (B) 3-D super-resolution images of 12 representative *B. anthracis* treated with PZN-Cy5. Scale bars, 1 µm.
**Figure 40.** DBSCAN cluster analysis on individual cells

Three examples of representative cells having PZN clusters are shown. Individual clusters are analyzed, yield their sizes. Different clusters are plotted in different colors (below).


Appendix

RIG-I Clusters in Granule under various Stress Conditions including Viral Infection

Differentiating real clusters from artificial ones, by frame number distribution (FND)

In this supplementary document, we will discuss several approaches to distinguish artificial clusters from real clusters. It has been previously reported that super-resolution microscopy based on on-off blinking and switching behavior can lead to artificial clustering [1]. In our case, we used image reconstruction algorithm that would reduce multiple counting of the same molecule [2]. However, this algorithm relies on the accuracy of spot recognition in each single frame to within 1 pixel (100nm) range, and continuing appearance of a single molecule over consecutive frames. Thus such an algorithm can fail in the presence of dim spots or overlapping spots in each frame. Therefore a quantities evaluation of the possibility of clustering artifact is desired.

In the previous report, a negative and a positive control were compared by measuring which frames would contribute to generate an identified cluster. A negative control case showed each final cluster is mostly composed by spots in nearby frames, indicating concomitant spatial, temporal and artificial clustering. In contrast, the positive control case showed each final cluster is mostly composed by spots appearing homogeneously over the entire duration of the data acquisition. We adapted this idea of measuring the “width and continuity” of the frame number distribution of appearing spots to form a cluster over the entire frames (FND for short), to verify our observation of RIG-I clustering. By characterizing FND of a certain cluster by its continuity and length, we can decide if that cluster is artificial or real[1]. Then we used various approaches to identify artificial clustering behaviors. By utilizing the identified artificial clusters as negative
controls, we will attempt to find the best parameters for DBSCAN clustering analysis. In the various investigations of this chapter, we used five sample images (Fig. A1).

Firstly, each cluster of an AVG induced by poly I:C was investigated (Fig. A2A). Figure A2B shows three histograms of counted number of spots appearing in each frame window of 2,000 frames, for three exemplary clusters. Then we super-imposed such histograms for RIG-I clusters in each of three different cases, that is, in an AVG, in cytoplasm upon poly I:C transfection, and in a 2nd antibody only control (Fig. A3).

**Counting empty frames**

A simple way to estimate the width of FND is just counting the nonempty frames in FND. 40 RIG-I clusters in an AVG are composed by spots appearing in most of the frames (19.26% frames were without spots, Fig. A3A, detailed description of how this value was obtained is described in Fig. A3), while 40 RIG-I clusters in the cytoplasm have more empty frames (29.54%, Fig. A3B). However 40 of the 2nd antibody control case clusters had many empty frames (58.5%, Fig. A3C). This means that a significant portion of the 2nd antibody control clusters are artificial, while RIG-I clusters in AVGs have the least number of artificial clusters and RIG-I clusters in cytoplasm are mixed with artificial ones.

**Gaussian fitting assuming a normal distribution of each cluster**

Counting the empty frames is one way to evaluate FND, but this lacks the details of the distribution. For instance, a cluster could be composed by one spot in every frame, except a few frames with many numbers of spots. In this case this cluster might be an artificial one with a non-blinking junk molecule within it, but the above analysis cannot rule out such a case. Thus we conducted Gaussian fitting to each FND assuming each one is following normal distribution (Fig. A4A). Then from each fitting, we could obtain the full width half maximum (FWHM) of each distribution per cluster. (Note that this fitting is not a direct fitting to the histogram. We will discuss about it shortly.) When we put the results for the three cases all together (Fig. A4B). This shows that RIG-I clusters in AVGs or cytoplasm have higher FWHM than the 2nd antibody control, again indicating clusters in the 2nd antibody control are mostly artificial, while RIG-I clusters in AVGs and cytoplasm are not.
However this analysis contains problematic cases (Fig. A4C). Here the fitting does not truly give the right width of the distribution, since the empty regions didn’t affect much to the evaluation of FWHM. It is because our fitting so far was based on the assumption that FND follows normal distribution. Thus in a case like this when the FND is discontinuous in the middle, this fitting does not work well.

**Fitting a single Gaussian function to FND**

Then we tried fitting the histogram directly by Gaussian function. However single Gaussian fitting does not work well (Fig. A5A), and this leads to distinctively high FWHM values for RIG-I in AVGs, while RIG-I in cytoplasm has no peak in the histogram (Fig. A5B) and 2nd antibody case is biased to the lowest FWHM. Such a case is hard to characterize and such extreme contrast among the three cases suggests analytical errors.

**Fitting four Gaussian function to FND**

Then we applied four Gaussian fittings to FND for each cluster, and it showed a clear advantage over a single Gaussian fit (Fig. A5C). Then the FWHM of each cluster histogram was redefined as the sum of the four FWHMs from those four Gaussian functions. Four Gaussian functions generally fit to a count histogram more precisely (Fig. A6). Then we got the new FWHM count plot for the three cases (Fig. A7A). The FWHM histogram of the 2nd antibody control case has a single peak around 15000 frames, while RIG-I clusters in AVGs and cytoplasm have smaller peaks in the similar FWHM range with the 2nd antibody control, and higher peaks around 30,000 frames. This indicates that some of the identified RIG-I clusters can come from artifacts, but the majority of them would come from real clusters. This result is more consistent with the first analysis of counting empty frames. Then we also used the same approach to analyze TIAR clusters from AVGs and cytoplasm (Fig. A7B), and the result was plotted together with the same 2nd antibody control case from Figure A7A. This indicates that most TIAR clusters are coming from detection artifacts as their FWHM distribution tends to follow similar trends with the 2nd antibody control case, having a single peak at lower FWHM.

**Binning free approach**
Even though the last result may evaluate the validity of clusters detected, this method depends on binning of the data to form histograms to start with. Therefore we were seeking a way to analyze the data without the necessity of binning, and found an alternative approach. Instead of binning the count per frame number windows, the number of spots appearing each frame was accumulated to give accumulated count over frame numbers. (Fig. A8) Then for the case of a real cluster, the accumulated count will gradually go up (Fig. A8A). However for an artificial cluster which is composed by spots appearing in nearby frames would result an abrupt jump in the accumulated count (Fig. A8C). Then we summed the area over and below the straight line

\[ y = \frac{\text{Total number of spots detected to form a cluster}}{\text{Total number of frames}} x \]

To make a fair comparison between different cases with different total number of spots per cluster, we divided this area by the total number of spots per cluster. To have more RIG-I clusters into consideration, a bigger RIG-I cytoplasm area (having 633 clusters) was used.

Then every cluster in three different cases were plotted together, in a plot of (Deviation area)/(number of spots per a cluster) against number of spots per cluster (Fig. A9). (Note that here “number of spots detected to form a cluster”=”number of spots per a cluster.” We often times included “to form” phrase to emphasize that we are calculating based on the original frame numbers of spots inside of a cluster for this analysis.) Then these three cases can be easily identified as three distinct groups in this plot, indicating RIG-I clusters in an AVG have the least artifacts, while 2nd antibody control has highest artifacts, and RIG-I clusters in cytoplasm are mixed.

Then we conducted the same analysis for TIAR case (Fig. A10). TIAR in cytoplasm distribution is within the error range of 2nd antibody control for TIAR, so TIAR clusters in cytoplasm are as artificial as the 2nd antibody control. However the center of TIAR clusters in AVGs is away from the error range of other centers (Fig. A10) and this may rise a question of how much portion of TIAR clusters are real. To address this question in the perspective of more real clusters of RIG-I, we plotted the centers with error bars only for RIG-I and TIAR together (Fig. A11). Then RIG-I clusters in an AVG has average normalized deviation area as 2452(±124), 37% less than the case of TIAR clusters in AVGs (3916(±180), Fig. A11). This
does not eliminate the possibility of existence of real clusters of TIAR, and this analysis does not provide further details of how much percentage of real clusters TIAR case has. Thus for more quantitative comparison, the previous approach of four Gaussian functions fitting to FND would work better, though it needs binning of the data.

**Searching for the best parameters for the cluster analysis**

As discussed above, the majority of RIG-I clusters in an AVG are likely to be real, while 2nd antibody clusters or TIAR clusters (in both AVGs and cytoplasm) would be mostly artificial. Then we attempted to use these contrasting characteristics to find the best parameters for our DBSCAN cluster analysis. In most parts of this section (except Fig. A12A~D), we used the same images as in Figure A1, the top left as the positive case (RIG-I in an AVG), and the top right (2nd antibody control) the bottom left (TIAR in AVGs) as negative controls.

As in the Materials and Methods, DBSCAN algorithm requires two parameters, Npts and Eps. Initially those parameters were chosen empirically (Fig. A12). Firstly, we tried various parameters in a broad range, to be away from extreme cases. For example, too big Npts value detects only a few clusters (Fig. A12E bottom right), or too small Eps hinders forming and growing clusters. Then we found the range of Npts=5~10 and Eps=33~33nm would be reasonable to search for the set of best parameters (Fig. A12E). We plotted the four main outputs of DBSCAN algorithm, i.e. the average diameter of clusters, the average number of localizations included in clusters, the number of clusters and the percentage of localizations included in detected clusters, against various parameters in the range, for RIG-I clusters in seven AVGs induced by poly I:C (Fig. A12A~D). The first two outputs are related to the final characteristics of the clusters, which are unknown yet. However the last two parameters can be related to our visual feedback of determining clusters. Then we found varying Eps didn’t change the number of clusters much (Fig. A12C), so chose the middle value, Eps=37. Also we could observe the number of clusters converge at Npts=7 for varying Eps values, and often times we could observe similar trends to other RIG-I clusters in AVGs. Thus we chose Npts=7, and this set of parameters, (7, 37nm) also matched well with visual feedback (Fig. A12E, A15). Thus they were used throughout the main results in this report.
However we can approach this pursue more analytically, utilizing the 2\textsuperscript{nd} antibody control images and TIAR images as the negative controls for clustering, as discussed previously (Fig. A7B). We can search for the best parameters that can detect the least “clustering” from the negative control cases (2\textsuperscript{nd} antibody and TIAR), while detecting the most “clustering” from the positive case (RIG-I in an AVG). Here “clustering” can be interpreted as either the percentage of clusters, or the number of clusters detected.

This task can be simplified as an equation of finding a variable $x$ that can maximize the “contrast” between two functions, $f(x)$ and $g(x)$, where $f(x) > g(x) > 0$. Here “contrast” is defined as maximizing $f(x)$ while minimizing $g(x)$. More specifically, we define that $x$ makes the greatest contrast between $f(x)$ and $g(x)$ when $x$ satisfies both of the following functions.

$$\Delta(x) := f(x) - g(x)$$

$$r(x) := \frac{f(x)}{g(x)}$$

This concept is visualized in Figure A13. Maximizing only one of $\Delta(x)$ or $r(x)$ is not good enough since there can be occasions when one of them does not change much while the other changes much. Thus $x$ that makes both $\Delta(x)$ and $r(x)$ maximized at the same time is what contrasts $f(x)$ and $g(x)$ the most.

We plotted the test case (RIG-I in an AVG) as well as the control cases (2\textsuperscript{nd} antibody, and TIAR in AVGs) in grayscale plots for their percentage of clustering (Fig. A14A) or the number of detected clusters (Fig. A14B). Then we either obtained the differences (Fig. A14, the second column) or the ratios (Fig. A14, the third column) between the test and control cases. Then out of eight grayscale maps as outputs, (Fig. A14, the second and third columns) six of them were maximum at (10, 33nm). Thus (10, 33nm) was tested to see if this set of parameters can actually suppress detecting artificial clusters better than (7, 37nm).

Firstly, we compared the detected clusters by direct visualization (Fig. A15). (10, 33nm) is a more strict condition than (7, 37nm), detecting 124 clusters instead of 159 clusters out of an AVG (Fig. A15A, right). Some of the real clusters might be removed too, but our visual feedback is limited to judge this.
On the other hand, (10, 39nm) which gave the more contrasts than (10, 33nm) for two outputs out of eight (Fig. A14A, the second column), caused artificial merging of distinct clusters (Fig. A15B, middle). Thus (10, 39nm) was not tested further.

Secondly, we plotted FWHM count histogram for RIG-I and TIAR clusters in AVGs or cytoplasm, along with 2nd antibody controls, from four Gaussian fittings to each FND of clusters identified by (10, 39nm) (Fig. A16). Unlike the previous case (Fig. A7A), RIG-I in AVG does not have a peak to lower FWHM, but has only a single peak at high FWHM. This implies new parameters could eliminate certain portion of artificial RIG-I clusters. RIG-I in cytoplasm still has two peaks, meaning mixed population of artificial and real clusters. For TIAR, the new parameters detected very few TIAR clusters (9 in AVGs, 5 in cytoplasm. Previously 161 in AVGs, 44 in cytoplasm), making them hard to characterize. However 2nd antibody clusters were still detected, and have a single peak at lower FWHM. This means the new parameter could avoid detecting artificial clusters from a more uniform case (TIAR), but 2nd antibody only can still give artificial clusters to be detected, even though the detected number is less (55 detected, previously 118).

Thirdly, we plotted the normalized deviation area against the number of spots per cluster, under the new analysis by (10, 33nm) (Fig. A17). RIG-I clusters both in and AVG and in cytoplasm showed distinct population as previously observed (Fig. A17A, A9). However TIAR clusters all fall into the error range of the 2nd antibody case (Fig. A17B, C), unlike the previous case where TIAR clusters in AVGs had a distinct population from others (Fig. A10, A11). This implies that (10, 33nm) could effectively eliminate artificial clusters of TIAR, that were otherwise detected by other sets of parameters.

The above two results (Fig. A16, A17C) collectively support the idea that (10, 33nm) could eliminate artificial TIAR clusters as well as a certain portion of artificial RIG-I clusters and 2nd antibody clusters. This concludes (10, 33nm) is the best set of parameters for the given dataset.

**RIG-I clustering in AVGs are also observed by structural illumination microscopy (SIM)**

To further test if RIG-I clustering observed was an artifact by our super-resolved single-fluorophore microscopy, we took images of RIG-I by structural illumination microscopy (SIM, Zeiss Elyra S1 SR-SIM) (Fig. A18). SIM images of RIG-I also verified clustering in AVGs by poly I:C transfection.
Figures

Figure A1. Five cases analyzed for determining the degree of artificial clustering. RIG-I images in an AVG and the cytoplasm area (top left two panels), a control case without the primary antibody, but with the same secondary antibody used for RIG-I detection (top right), TIAR in five AVGs, TIAR in the cytoplasm area (bottom left two panels) and another control case without the primary antibody but with the same secondary antibody used for TIAR detection (bottom right) went through various analysis. For RIG-I, only one AVG was analyzed since it provided enough number (157) of clusters. However since TIAR is less clustered, five AVGs were analyzed together for TIAR-clustering. There are 161 TIAR clusters in five AVGs. In all the cases, cells were transfected by poly I:C. Images have different contrasts for the best visualization.
Figure A2. Occurrence of spot detection per frame windows in RIG-I clusters in an AVG

(A) A three-dimensional view of RIG-I clusters in an AVG formed by poly I:C transfection (B) Three exemplary histograms from three RIG-I clusters from (A). The number of spots appearing in 2,000 frame windows is plotted across the total 30,000 frames.
Figure A3 (cont.)
Figure A3. Superimposition of occurrence histograms and normalization for three cases. “Frame window” is defined by a matrix of histogram values (Fig. A1B) sized as (Total number of frames)/(frame window size)X(number of clusters)=(30,000/2,000)X40 = 600 entries, since the histogram bin size is 2,000 frames. Then each cluster can have zero values in these entries. The final percentage is calculated by counting zeros in all the entries of the frame windows for 40 clusters per each case, then divided by the total number of the entries of the frame windows. (A) A superimposition of spot detection occurrence per 2,000 frame windows for 40 clusters in an AVG by poly I:C transfection, and its normalized plot. (B) A superimposition of spot detection occurrence per 2,000 frame windows for 40 clusters in a cytoplasm area of RIG-I, and its normalized plot. (C) A superimposition of spot detection occurrence per 2,000 frame windows for 40 clusters in a cytoplasm area of secondary antibody nonspecific binding. Here the secondary antibody is the same one against RIG-I primary antibody, and its normalized plot.

\[58.5\%\] of entries of frame windows were blank (i.e. most of frame window had at least one localization)
Figure A4. Determining FWHM by Gaussian fitting to each distribution of spots appearing per each frame window of 2,000 frames, assuming normal distribution

(A) One example of Gaussian fitting to the frame number distribution (FND) of spots appearing for a RIG-I cluster in an AVG. The curve is not a direct fitting to the histogram, but a fitting to the distribution by assuming normal distribution. (B) Count of FWHM of FNDs for three different cases as RIG-I clusters in an AVG, RIG-I clusters in cytoplasm, and secondary antibody control without the primary antibody. Here the secondary antibody is the same one used for the other two cases. (C) Another example of Gaussian fitting to a distribution of a RIG-I cluster in an AVG. The curve does not reflect the true distribution of frames where spots appeared.
Figure A5. Single Gaussian fitting vs. four Gaussian fittings to fit FND histograms

(A) One example of direct Gaussian fitting to a histogram of FND, with 2,000 frames of binning, for a RIG-I cluster in an AVG. (B) Count of FWHM for three different cases as RIG-I clusters in an AVG, RIG-I clusters in cytoplasm, and secondary antibody control without the primary antibody. Here the secondary antibody is the same one used for the other two cases. FWHMs are biased to smaller values. (C) Four Gaussian fittings to the same distribution histogram give much better fitting.
Figure A6. Examples of four Gaussian functions fitting into FND histograms for six RIG-I clusters from an AVG.
Figure A7. FWHM count histograms for RIG-I and TIAR from AVG or cytoplasm, along with 2nd antibody control case are from four Gaussian fittings to each FND. (A) Comparison for RIG-I: The 2nd antibody control case has a single peak, but other cases have double peaks. Peaks at smaller FWHM arise from the clustering artifacts, but peaks at higher FWHM are due to real clustering. (B) Comparison for TIAR: TIAR clusters along with the same 2nd antibody used for TIAR staining have single peaks only, implying most TIAR clusters (both in AVGs and cytoplasm) are artificial.
Figure A8. Examples of three clusters in different conditions and the measure of continuity of FND without binning of the frames (A) A RIG-I cluster in an AVG shows continuous appearance of spots over the whole frames, which translates into the smallest area over and below of the straight line connecting (0,) to the (Total frame number, Total number of spots). We divided the area by the total number of spots for normalization, and termed it “normalized deviation area.” (B) A RIG-I cluster in the cytoplasm is composed by continuously appearing spots with occasional blank frames in the middle, resulting higher normalized “deviation area” around the straight line (the right panel). (C) An artificial cluster is composed by spots in nearby frames only, resulting the highest normalized deviation area (the right panel).
Figure A9. Plots of “total number of spots in FND to form a cluster” versus “normalized deviation area” show clear contrasts between the three cases. Each symbol represents a single cluster. (A) RIG-I clusters in an AVG. Each data point represents a single RIG-I cluster. (B) RIG-I clusters in the cytoplasm. (C) 2nd antibody clusters with their centers and (D) All the three cases are combined together. The three arrows indicate where the centers are for the three cases. The magenta Square and lines represent the center and standard error for RIG-I clusters in an AVG, the yellow square and lines are for RIG-I in cytoplasm, and the cyan square and lines are for 2nd antibody clusters as pointed by arrows. These three populations are clearly distinctive for their centers and errors.
Figure A10. Plots of “total number of spots in FND to form a cluster” versus “normalized deviation area” show less contrast between the three cases for TIAR. Each symbol represents a single cluster. (A) TIAR clusters in an AVG: Each data point represents a single RIG-I cluster. (B) TIAR clusters in the cytoplasm. (C) 2nd antibody clusters with their centers and (D) All the three cases are combined together. The violet square and lines represent the center and standard error for TIAR clusters in an AVG, the orange square and lines are for TIAR in cytoplasm, and cyan square and lines are for 2nd antibody clusters as pointed by arrows. These three populations are not as distinctive as for RIG-I.
Figure A11. Only the centers (averages) and standard errors for the three cases, for RIG-I and TIAR, are plotted together, taken out from Fig. A9 and S10. RIG-I clusters are distinct from 2nd antibody controls and TIAR in cytoplasm, which are likely to be artificial (Fig. A7B). TIAR clusters locate in between.
Figure A12. Searching for the best parameters for detecting clusters empirically. Seven RIG-I AVGs by poly I:C were gone through DBSCAN cluster analysis by 42 sets of parameters (Npts=5~10, Eps=33~39nm). (A) The size (average diameter) of clusters (B) The average number of localizations in clusters (C) The number of clusters (D) The percentage of localizations within detected clusters is plotted in different sets of Npts and Eps. (E) Examples of clusters detected by various sets of Npts and Eps parameters. Different colors represent different clusters detected. Parameters beyond the range of (A–D) were also tested. The notation convention: (N, L nm) means Npts=N, Eps=L nm for DBSCAN analysis.
Figure A13. A simple model of defining “contrast” between two functions. (A) In a case when the difference of two functions is the same, $x = b$ will give the maximum contrast between two, since $f(b)/g(b) > f(a)/g(a)$, even though $f(a) > f(b)$. (B) In a case when the ratio between two function is the same, $x = b$ will give the maximum contrast since $f(b) - g(b) > f(a) - g(a)$, even though $g(a) < f(a)$. 
Figure A14. Searching for the best parameters for detecting clusters analytically. RIG-I clusters in an AVG, 2nd antibody control for RIG-I, and TIAR clusters (the latter two are controls) in AVGs were analyzed as described in Fig. A12A. (A) The percentages of clustering were plotted by grayscale maps for the three cases (the first column). To obtain the maximum contrast between the test (RIG-I in an AVG) and control cases, we either subtract the control cases from the test case (the second column) or divided the test case by the control cases (the second column). Then we plotted the results by grayscale maps again. (B) The same analysis as (A), except that the number of cluster was the measure. In almost all cases, (10, 33) gave the highest contrast.
Figure A15. How different parameters affect detection and merging of the clusters. As examples, an RIG-I AVG is displayed under three different sets of parameters used for cluster analysis for comparing the effects of changing parameters. Black points represent localizations that are not included in detected clusters. Different clusters are depicted as groups of different colors. (7, 37 nm) was what we used in the main Results of this report. (10, 33 nm) could be the best potential parameter according to Fig. A14. (10, 39 nm) case was also included as an intermediate. (A) Different sets of clusters can nullify certain clusters. For the five clusters circled for (7, 37 nm) case (left), three of them are nullified by (10, 39) case (middle). Then these two are nullified also by (10, 33) case (right). (B) Different sets of clusters can merge or separate clusters. For the two sets of different clusters in rectangles for (7, 37 nm) case (left), they are merged together in (10, 39) case (middle). However (10, 33) case separate them into individual clusters again (right).
Figure A16. FWHM count histograms for RIG-I and TIAR from AVG or cytoplasm, along with 2nd antibody control case are from four Gaussian fittings to each FND, obtained by (10, 33nm) parameters. (A) Comparison for RIG-I: The 2nd antibody control case has a single peak to lower FWHM, while RIG-I case has a single peak also at higher FWHM, unlike the two peaks in Fig. A7A. RIG-I in cytoplasm has two small peaks around 2nd antibody peak and RIG-I in AVG peak, implying mixed population of artificial and real clusters. (B) Comparison for TIAR: Very few TIAR clusters were detected, making them hard to characterize. On the other hand, 2nd antibody case has a single peak at lower FWHM, being consistent with (A).
Figure A17. Plots of “total number of spots to form a cluster” versus “normalized deviation area” were obtained for RIG-I and TIAR cases respectively, using the new parameters, (10, 33nm). (A) Both RIG-I in an AVG and RIG-I in cytoplasm distributions show distinct populations from the 2nd antibody control case. (B) There were very few TIAR clusters obtained, and even so, their distributions are within the error range of the 2nd antibody case. (C) Only the centers and standard errors for all the cases are plotted together. RIG-I clusters are distinct from other cases as before, but TIAR clusters in AVGs fall into the error range of TIAR in cytoplasm and 2nd antibody controls, implying that the new set of parameters could effectively eliminate artificially detected TIAR clusters in AVGs. The 2nd antibody control with black square center is for RIG-I, and the 2nd antibody with red triangle is for TIAR.
Figure A18. RIG-I imaging by structural illumination microscopy (SIM). RIG-I clusters in AVGs in a cell treated by poly I:C transfection are imaged by structural illumination microscopy (right), with an image obtained by regular microscopy (left). White rectangle areas are zoomed in (bottom).
References
