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## **Chapter 23**

### **Defining assembly pathways by fluorescence microscopy**

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## Summary

Bacterial secretion systems are amongst the largest protein complexes in prokaryotes, and display remarkably complex architectures. Their assembly often follows clearly defined pathways. Deciphering these pathways not only reveals how bacteria accomplish to build these large functional complexes, but can provide crucial information on the interactions and subcomplexes within secretion systems, their distribution within the bacterium, and even functional insights. The emergence of fluorescent proteins has provided a new powerful tool for biological imaging, and the use of fluorescently labeled components presents an interesting method to accurately define the biogenesis of macromolecular complexes. Here, we describe the use of this method to decipher the assembly pathway of bacterial secretion systems.

**Key Words:** Fluorescence microscopy, biogenesis, secretion systems, fluorescently labeled proteins, macromolecular complexes, epistasis experiments, subcellular localization

**Running head:** Protein dynamics by fluorescence microscopy

## 1. Introduction

Bacterial secretion systems are macromolecular machines that mediate the transport of proteins between bacteria or from bacteria to eukaryotic cells (*1,2*). These complexes incorporate one or multiple copies of a large number of different proteins that are recruited in a hierarchical order. Important insights into the assembly of secretion systems were obtained by the purification and visualization of assembly intermediates, either in strains lacking certain components of the system, or upon overexpression of defined components (*3-10*). However, the often low number of stable intermediates and the difficulties in obtaining and visualizing these have limited the utilization of this approach. Increasingly, the assembly of secretion systems is therefore deciphered based on the localization of fluorescently labeled subunits. Variations of this approach have been applied for a variety of secretion systems

including the Tat system (*11,12*), the type II secretion system (T2SS) (*13,14*), the T3SS (*15,16*), the T4SS (*17*), or more recently the T6SS (*18*). The rationale is that a fluorescently labeled component forms distinctive fluorescent foci where the secretion system resides, in wild-type cells and in strains lacking components that are not required for its assembly, but will have a diffuse fluorescent pattern when components responsible for its recruitment are missing. Prerequisites for this approach are the genetic amenability of the bacterium to create fluorescent fusion proteins, and a specific distribution of the secretion system(s) within the bacterium (*see Note 1*). Beyond studying the kinetics of secretion system assembly in live bacteria, this method can be used to obtain a detailed description of the assembly pathway by visualizing the labeled subunits in strains lacking other components of the secretion system. In this chapter, we describe a generally applicable approach to decipher the assembly pathway of secretion systems using fluorescently labeled proteins.

## 2. Materials

### 2.1 Strains

1. Strain(s) expressing a chromosomal fusion of a fluorescent protein to the target protein of interest, e.g. superfolder green fluorescent protein (sfGFP) fused either to the N- or C-terminus of the target protein (*see Notes 2 and 3*).
2. Additional deletions of other components of the secretion system in the strain background mentioned above, to allow the investigation of the order of assembly.
3. Recommended: Untagged strain as a control for autofluorescence.
4. Recommended: Strain expressing the fluorescent protein in the cytosol from plasmid as a control (*see Note 4*).

### 2.2 Sample preparation

1. Incubator shaker
2. Spectrophotometer

3. Culture medium: standard culture medium and appropriate antibiotics for overnight incubation and growth of bacteria, e.g. Luria Broth (LB) or M9 minimal medium (*see Note 5*).
4. Microscopy buffer: non-fluorescent minimal medium or imaging buffer, e.g. phosphate buffered saline (PBS) (*see Note 6*).

### 2.3 Microscope slide preparation

1. Low-melting agarose (or agar), commercially available.
2. Microscopy slides and cover slips compatible with the used microscope. Standard sizes include 75\*25\*1 mm glass slides and 22\*22 mm cover slips, with No.1 (0.13-0.16 mm) being the most commonly used thickness.
3. Microwave oven to prepare agarose solution.

### 2.4 Image acquisition

1. Automated inverted epifluorescence microscope with 60× or 100× objective (*see Note 7*).
2. Optical filters for visualizing fluorescence, e.g. ET-GFP filter set (Chroma 49002) for visualizing GFP fluorescence and ET-mCherry filter set (Chroma 49008) for visualizing mCherry fluorescence.
3. Dichroic mirrors compatible with the used fluorophores and filter sets.
4. Incubation chamber for microscope stage, where required.

### 2.5 Software for image processing

1. Proprietary software often pre-installed on microscopy controller, commercial software like Adobe Photoshop, or open-source solutions like ImageJ, a widely used and adaptable open source image processing program (<http://rsb.info.nih.gov/ij/>) (**19**).

## 3. Methods

As the protocols for propagation of bacteria and induction of secretion systems greatly vary, we aim to provide a general protocol, which can be adapted to the specificities of the studied secretion system.

All buffers and solutions should be prepared using ultrapure water at room temperature.

### 3.1 Preparation of bacteria and setup of microscopy equipment

1. Streak bacteria from  $-80^{\circ}\text{C}$  stock onto LB agar plates containing the required additives and antibiotics and incubate at the required temperature (e.g.  $37^{\circ}\text{C}$ ), until single colonies are visible (usually 12-36 h).
2. Inoculate overnight cultures of bacteria with single colonies from agar plates and grow in shaking incubator at the required temperature and agitation.
3. On the next day, determine the optical density at 600 nm wavelength ( $\text{OD}_{600}$ ) of the overnight culture and inoculate a main culture to  $\text{OD}_{600}$  that is suitable for the expression of the analyzed secretion system (a culture volume of 5 mL is sufficient, and an  $\text{OD}_{600}$  of 0.1 is a good starting point for many systems).
4. Incubate bacteria in a shaking incubator until they reach early stationary phase (usually one to two hours).
5. Induce secretion system according to standard conditions (e.g. temperature shift, addition of inducer).
6. In the meantime, prepare a 1.5% solution of low-fluorescence agarose in microscopy buffer (*see Note 8*). While less than 100  $\mu\text{L}$  of agarose solution is required per strain, a higher volume (20-50 mL) is easier to prepare, and the agarose concentration is less influenced by evaporation. Add the agarose to the buffer, which is then carefully brought to boiling in a microwave oven. Be aware of the possibility of delays in boiling and use precaution. Check that the agarose is completely dissolved and allow to cool to approximately  $55^{\circ}\text{C}$ . After cooling, add any required additives (antibiotics, inducers) (*see Note 9*).
7. Prepare a thin pad of 1.5% agarose on a microscopy slide (Figure 1) (*see Note 10*).
8. Harvest exponentially growing cells ( $\text{OD}_{600} \sim 0.8-1$ ) by centrifugation ( $2,400 \times g$ , 4 min; these values depend on the bacterium). Resuspend in imaging buffer and recentrifuge

once, using the same settings, before resuspending in imaging buffer to an  $OD_{600} \sim 2$  (*see Note 11*).

9. Remove cover slip from agarose patch prior to spotting the bacteria, and wait until no more liquid areas are visible on the surface of the agarose patch (usually 1-5 min).
10. Spot bacteria by either of the following two methods:
  - a. Pipette 1-2  $\mu\text{L}$  of resuspended bacteria into the center of the agarose or agar patch without damaging the patch itself, let dry for about one to two minutes (*see Note 12*), and carefully cover with a cover slip.
  - b. Spot 1-2  $\mu\text{L}$  of resuspended bacteria onto a cover slip and carefully cover with an agarose or agar pad (Figure 2).

### 3.2 Microscopy

1. Place a drop of immersion oil onto the center of the cover slip, and flip the slide, so that the cover slip faces the objective. Carefully insert the slide into the microscope and bring the lens into contact with the immersion oil.
2. In phase contrast or differential interference contrast (DIC) mode, slowly decrease the distance between the lens and the cover slip, until bacteria are visible (*see Note 12* in case large numbers of detached or swimming bacteria are visible).
3. Adjust Koehler illumination of microscope for optimal phase contrast or DIC images (*20*).
4. To determine which phase contrast / DIC plane corresponds to the best fluorescence plane, run an automated z stack of phase contrast / DIC and fluorescence images (*see Note 13*). For bacteria with a diameter of about 1  $\mu\text{m}$ , z stacks containing 10-20 planes with  $\Delta z = 100$  nm yield sufficient coverage.
5. Capture phase contrast and fluorescence micrographs:
  - a. For kinetic studies: every 30 sec with a minimal exposure time to minimize bleaching and phototoxicity effects (*see Note 14*)
  - b. For the determination of the assembly pathway: single micrograph or z stack image (*see Note 15*) in WT and mutant strains.

### 3.3 Image processing

1. Phase contrast and fluorescence images can be adjusted and merged using ImageJ or equivalent software (*see Note 16*).
2. Slight movements of the whole field during the time of the experiment can be corrected by registering individual frames using StackReg plug-in in ImageJ (<http://fiji.sc/StackReg>).
3. Blurring of the image can be reduced by deconvolution, a mathematical post-imaging process which removes or reassigns the fraction of detected photons caused by out-of-focus structures. This is especially useful when three-dimensional information from z stacks is available. Care should be taken not to mistake deconvolution artifacts for clustering, and negative controls are mandatory when applying deconvolution.
4. Detection and quantification of bacteria and fluorescent foci can be performed in ImageJ or using the Oufiti software package (formerly known as MicrobeTracker, <http://www.oufti.org/> (**21**)).

### 3.4 Determination of the assembly pathway

1. To build an assembly pathway from the aforementioned data, determine the presence and number of foci per cell in wild type and each mutant strains. Nucleating component(s) of the system are correctly localized in foci in strains lacking all other components of the secretion system. Subsequently assembled proteins correctly localize in foci in strains expressing all earlier assembled proteins. Finally, protein(s) that require the presence of all the other components of the system are recruited at the end of the assembly. Proteins which are recruited at the same time or interact together before being recruited to the system will show the same localization in mutant strains, and usually require each other to form foci.

## 4. Notes

Notes 1 to 4 correspond to the prerequisites for the applicability of this approach.

1. Most bacterial secretion systems have a sufficiently distinct localization within the bacterium that differs from the distribution of the free component. Should this not be the case, standard fluorescence microscopy cannot distinguish between the free and assembled state of the labeled component, and more sophisticated methods such as diffusion-based fluorescence correlation spectroscopy (FCS) or interaction-based Förster resonance energy transfer microscopy (FRET) have to be applied.

2. While most fluorescent proteins are relatively inert to interactions, their size of 25-30 kDa (22) can lead to cleavage or degradation of the fusion protein, and impede assembly or functionality of the tagged protein. Smaller alternatives, such as tetracysteine tags (23,24), require additional manipulation (25) and may also disturb the function of the protein (own unpublished observations). It is therefore essential to test expression level and stability of the fusion protein (by immunoblot), as well as the functionality of secretion system in the corresponding strains (by a functional assay). While fusions that influence the function may be perfectly fine tools to decipher assembly, this has to be corroborated by independent experiments. To maximize the chances of obtaining a functional fusion protein, both termini of the protein as well as internal flexible loops should be considered. Flexible linkers between the fluorophore and the secretion system component (e.g. a stretch of 6 – 15 amino acids with a high glycine content) have been shown to preserve functionality of the fusion protein. Chromosomal fusion proteins are preferred to avoid mislocalization due to overproduction of the protein or wrong timing and order of the expression of subunits. Moreover, chromosomal fusions allow to analyze the secretion system under close-to-wild type conditions. However, especially for C-terminal fusions, care has to be taken not to disturb the expression of downstream genes in the same operon, and it has proven helpful to repeat the genetic region upstream of the following gene.

3. Concerning the choice of fluorescent protein, many variants of GFP have been produced which vary in spectral properties, degrees of multimerization, folding rates and functionality in oxidizing environments, so it can be helpful to try different fusion proteins (22). Due to their fast folding, low multimerization tendency, and proper folding in the periplasm, sfGFP and mCherry are good starting points. mCherry has additionally been observed to retain functionality of the T3SS in cases where GFP fusions were non-functional (26 and unpublished results). Most bacteria also display considerably less autofluorescence in the red

spectrum; however, mCherry is less photostable than GFP, which might make it less suitable for time course studies.

4. While these control strains are not absolutely required, especially in the presence of good controls for the protein of choice itself (i.e. deletion of a protein required for its localization), they are immensely valuable to set up and test the microscopy pipeline.

5. M9 and similar buffers have the advantage that due to their low autofluorescence, they can be directly used in microscopy. This ensures constant external conditions for the bacteria and may eliminate the need for the washing step described in step 8.

6. The choice of imaging buffer is crucial to obtain reproducible results, as it will influence the bacterial metabolism and possibly the state of the secretion system to be analyzed. While PBS is a popular and easy-to-obtain imaging buffer, some bacteria show visible alterations in cell morphology in PBS within less than an hour. Preliminary experiments can reveal whether cell morphology and the distribution of secretion systems are affected in different imaging buffers.

7. The microscope must have a sufficient resolution and, most importantly, a high sensitivity to visualize and resolve the assembled proteins. A 100× objective is required for most distributions of secretion systems, although the formation of a polar spot or the distribution of few membrane-bound foci in large bacteria can be detected with a 60× objective. Sensitivity of the microscope is crucial, especially for low-stoichiometry components. The labeled protein has to be present in multiple copies within the complex to be detectable. In our experience, sensitive widefield microscopes can detect about ten molecules within a diffraction-limited spot over low background. For single-molecule detection, more sensitive methods, such as total internal reflection microscopy (TIRF) (27) or photoactivated localization microscopy / stochastic optical reconstruction microscopy (PALM / STORM) (28) have to be applied.

8. The pad can also be done with agar (instead of agarose). This can be especially useful for longer time course experiments, where bacteria can be incubated for one hour at optimal growth temperature prior to microscopy acquisition to allow cell division on a plane surface.

9. The agarose solution should be prepared or re-dissolved freshly before the experiment. The solution will stay liquid in a 55°C water bath; small aliquots can be kept in a tabletop incubator shaker for 1.7 mL reaction tubes (vigorous shaking is required to prevent solidification of the agarose in this case).

10. The depth of the pad can vary; however, the surface should remain as smooth as possible. The pad can be prepared using a microscopy slide and a cover slip, with spacers, using commercially available systems (e.g. GeneFrame) (Figure 1), alternatively using two microscope slides or prewarmed protein gel chambers for larger patches. The pad should be bubble free to facilitate the observation. Let the agarose solidify and dry at room temperature (>1 min, longer storage times are possible if the pad remains covered).

11. An OD of 2 leads to about 5% of the area being covered with bacteria (for *E. coli*; this obviously depends on the size of the bacterium), Increasing the OD will increase confluence leading to more cell-cell contact.

12. Depending on their surface and the properties of the agarose patch, bacteria may take some time to settle at this point. If a large part of the bacteria are still moving after some minutes, the volume of bacterial resuspension should be reduced and drying times increased. Don't dry the agarose pad at 4°C to avoid drifts during the observation.

13. To avoid saturation, strong photobleaching, or phototoxicity effects, all fluorescence images should be acquired with the minimal exposure time required to reach a sufficient signal/noise ratio. The optimal exposure time has to be determined for each protein; depending on the sensitivity of the microscope, exposure times of 20-100 ms for phase contrast or DIC, and 100 ms – 2 s for fluorophores are good starting points. Narrow band microscopy filters can also reduce photobleaching.

14. For time-lapse experiments, many microscopy systems allow to define fields of view (x, y, z, focus offset) that are stored and then automatically accessed by a motorized stage. The fields of view should be sufficiently apart to avoid cross-photobleaching (run preliminary experiment with long exposures to determine the area of bleaching, if required). Ten fields of view at an OD of about 2 usually yield a sufficient number of bacteria for further analysis.

15. Z stacks allow a more complete coverage of the bacterium, ensuring images that comprise the region of interest (often the center of the bacterium). In addition, the three-dimensional data yields information about the spatial distribution of the secretion systems within the bacterium, and allows better deconvolution of the images. However, imaging z-stacks leads to stronger photobleaching, and is therefore generally avoided in kinetic experiments. For kinetic experiments, keeping the focus is of particular importance, and hardware-based focusing systems or sealed plates can be of advantage.

16. To avoid the loss of raw data, the original images files should be preserved. Keep the low and high boundary below the background value and above the highest measured intensity, respectively, to prevent misinterpretation of the data. Within an experiment, these values should be kept constant after background correction.

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### **Figure legends**

**Figure 1** – Methods for the preparation of agarose pads

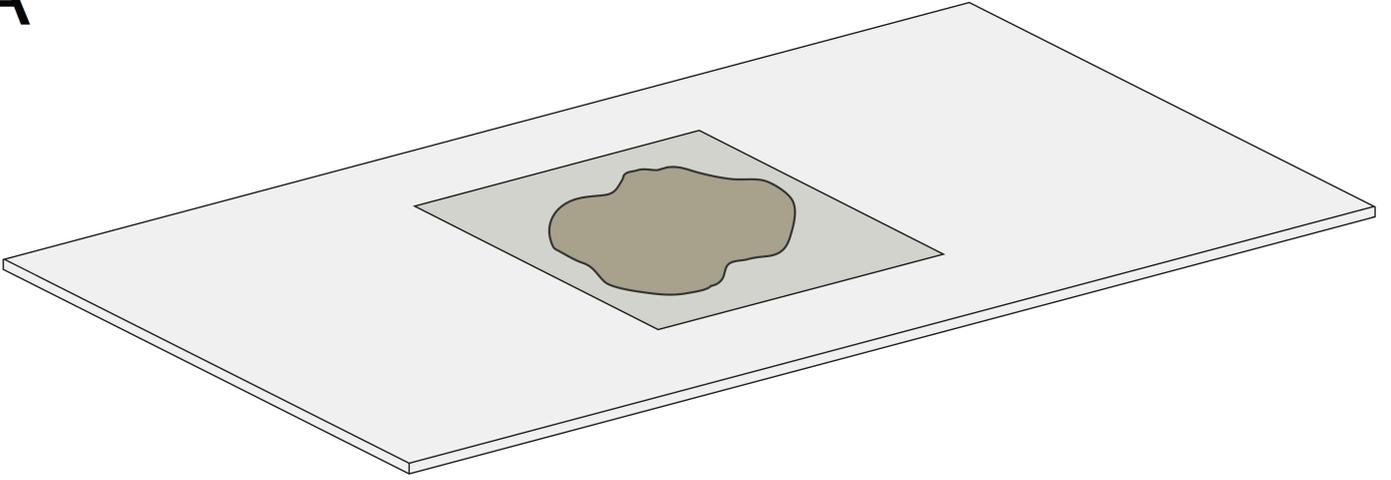
**A,** Approximately 50  $\mu\text{L}$  of agarose solution (beige) are transferred onto a microscope slide, and quickly covered with a cover slip (grey), which is then gently and evenly pressed onto the agarose to form an evenly distributed patch. This simple method is sufficient for quick imaging, but often leads to uneven or sloped agarose patches, which reduce image quality.

**B,** To ensure a more level surface of the agarose patch, the covering cover slip can be supported by two flanking cover slips.

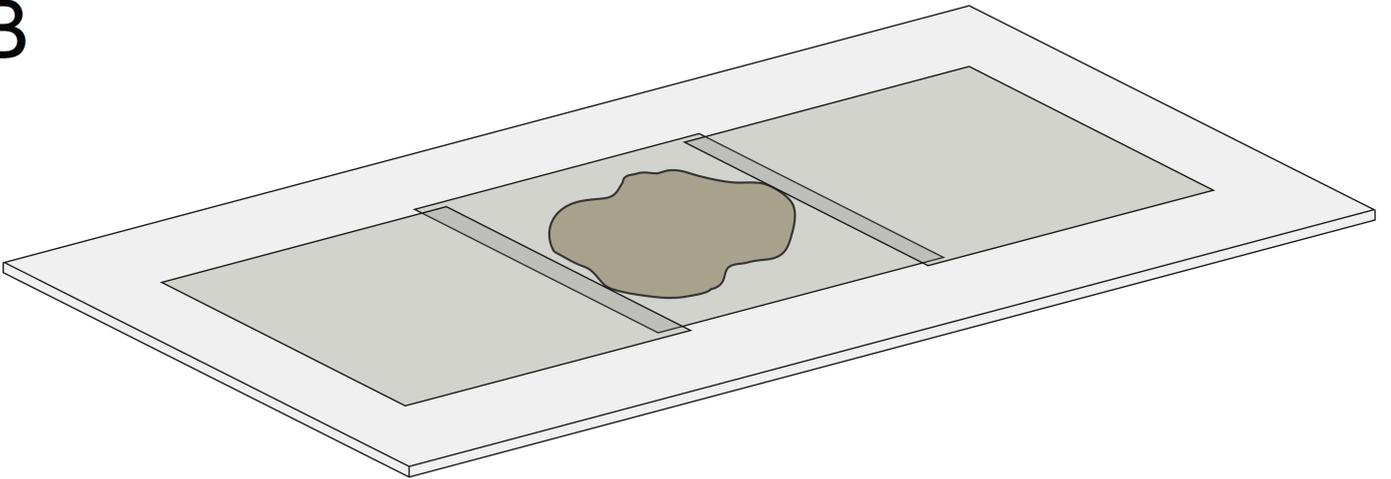
**C,** Double-sided tape or commercially available adhesives (e.g. “Gene Frame”, ThermoFisher) (rippled pattern) can be used to permanently adhere the cover slip to the sample. This prevents evaporation during imaging, which is very useful for longer experiments. However, the decreasing oxygenation of the sample should be kept in mind in this case.

**Figure 2** – Schematic representation of bacteria loaded onto cover slip and covered with agar pad.

**A**



**B**



**C**

