Revised paradigm of aquatic biofilm formation facilitated by microgel transparent exopolymer particles

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Transparent exopolymer particles (TEPs) are planktonic, organic microgels that are ubiquitous in aqueous environments. Increasing evidence indicates that TEPs play an active role in the process of aquatic biofilm formation. Frequently, TEPs are intensely colonized by bacteria and other microorganisms, thus serving as hot spots of intense microbial activity. We introduce the term “protobiofilm” to refer to TEPs with extensive microbial outgrowth and colonization. Such particles display most of the characteristics of developing biofilm, with the exception of being attached to a surface. In this study, coastal seawater was passed through custom-designed flow cells that enabled direct observation of TEPs and protobiofilm in the feedwater stream by bright-field and epifluorescence microscopy. Additionally, we could follow biofilm development on immersed surfaces inside the flow cells. Within minutes, we observed TEP and protobiofilm patches adhering to these surfaces. By 30 min, confocal laser-scanning microscopy (CLSM) revealed numerous patches of Con A and SYTO 9 staining structures covering the surfaces. Atomic force microscopy showed details of a thin, highly sticky, organic conditioning layer between these patches. Bright-field and epifluorescence microscopy and CLSM showed that biofilm development (observed until 24 h) was profoundly inhibited in flow cells with seawater prefiltered to remove most large TEPs and protobiofilm. We propose a revised paradigm for aquatic biofilm development that emphasizes the critical role of microgel particles such as TEPs and protobiofilm in facilitating this process. Recognition of the role of planktonic microgels in aquatic biofilm formation can have applied importance for the water industry.

Transparent exopolymer particles (TEPs) are intensely sticky, organic microgels, ranging in size from ~0.4 to >200 μm , present in large numbers in all aquatic environments. Since first described by Alldredge et al. (1), the ubiquity and multiple ecosystem functions of TEPs have been extensively documented in the oceanographic and limnological literature (2, 3).

TEPs and other microgel particles in marine and freshwaters are a part of a size continuum of organic matter that ranges from polymers through nanogels to microgels to very large marine (or lake) snow particles. Nano- and micro-sized porous gels composed of polysaccharides, proteins, and nucleic acids form from organic polymers and colloids in seawater by abiotic processes driven by electrostatic and hydrophobic bonding (4–6). TEPs can also derive directly from gelatinous envelopes surrounding algal cells, from bacterial mucous, or from degradation processes of marine or lake snow and other detrital material (7). Senescent or nutrient-stressed algae and cyanobacteria have also been shown to generate TEPs directly (8). Planktonic organic microgels such as TEPs may provide the scaffolding for “hot spots” of intense microbial activity (9, 10). These gel clusters frequently harbor extensive populations of bacteria (11, 12) (in this paper, the term “bacteria” refers to both bacteria and archaea) and larger microorganisms such as protista and algae (13).

Recently, TEPs have been implicated as an important factor in the development of aquatic biofilm (2, 14, 15). Like the extracellular polymeric substances (EPSs) that form the matrix of microbial

biofilms, these microgel particles are partly composed of polysaccharides with highly surface-active polymers of fucose and rhamnose (16). They are, thus, about two to four orders of magnitude stickier than phytoplankton or mineral particles and have a high probability of coagulation or surface attachment upon collision (17, 18). Therefore, TEPs are likely to play an important role in coating submerged surfaces and in the formation of aquatic biofilm (14, 15, 19).

Biofilm is defined as a sessile assemblage of complex microbial microcolonies, attached to a surface, and held together within a matrix of self-produced, predominantly mucopolysaccharide EPSs (20–22). The microcolonies are characterized by a basic architecture of multilayered, loosely packed bacterial cells encased in EPSs, separated by interstitial water channels that allow transport of nutrients, oxygen, chemical messengers, genetic material, and antimicrobial agents (22). Once established, biofilms are notoriously resistant to removal by treatments with chlorination, biocides, or antibiotics because of the protection provided by the multilayered EPS matrix. Therefore, much current, applied research is aimed at inhibiting either the outgrowth of biofilm forming bacteria or bacterial adhesion to sensitive surfaces.

These applied approaches are based on the following conception of aquatic biofilm formation: an initial, preconditioning phase, lasting from a few seconds to several hours, changes the chemical and physical characteristics of the surface (23–25). Dissolved organic polymers and colloids present in the overlying water immediately begin to adhere to the surface, forming a thin (<300-nm) “conditioning film” composed of large variety of adsorbed molecules: polysaccharides, proteins, lipids, and humic and nucleic acids (25, 26). Bacterial cells in the overlying water encounter the conditioning film and adhere to the surface. Cell adhesion is initially reversible, involving weak electrostatic forces and hydrophobic interactions. In this phase, bacteria still exhibit Brownian motion and are easily removed by application of mild shear forces. After several hours, most of the adhering bacteria become irreversibly attached through strong dipole–dipole forces, hydrogen and covalent ionic bonding, and hydrophobic interactions. The attached bacteria proliferate using dissolved organic matter as a nutritional source and are triggered to produce EPSs, eventually forming mature biofilm (20, 27, 28). Factors involved in the development of mature biofilm include bacterial quorum sensing (29, 30), nutrient availability (31), and cell death and lysis (32). Depending on environmental conditions, within hours to days after the initial irreversible adhesion,

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the organized structure of a mature biofilm develops. The process described above posits that the critical step for the establishment of biofilm is the successful, irreversible adherence of single bacteria to the substrate and assumes that the nutrition fueling bacterial growth in aquatic biofilm derives from dissolved organic matter within the overlying water.

In the present study, we followed the initial stages (minutes to hours) of biofilm development using an experimental flow cell system (Fig. 1). Our results confirm the hypothesis that TEPs, in particular large TEPs heavily colonized by bacteria and other microorganisms that we have termed “protobiofilm,” play a critical role in the initial stages of biofilm formation and significantly accelerate the rate of biofilm establishment. Based on the results of the present study, we propose a modified model of aquatic biofilm formation that takes into account the involvement of microgel particles such as TEPs in this process.

Results and Discussion

TEP and Planktonic Protobiofilm. Many studies have been published on the occurrence and ecosystem importance in aquatic environments of planktonic “hot spots” (2, 9, 10, 13). These are generally visualized as clusters of microorganisms held on and within a gel-like matrix and profoundly influence biogeochemical transformations within the water mass (10). Here, we propose the term “protobiofilm” to refer to planktonic microgel clusters within which extensive microbial outgrowth and colonization have occurred. Such particles display most of the characteristics of early developing biofilm, with the exception of being attached to a surface. Recently, the occurrence of *Legionella pneumophila* in 30–300- μm -thick “floating biofilms” at water/air interfaces was reported (33).

To study details of the involvement of TEPs and protobiofilm in the early development of biofilm, we used an experimental system based on custom-designed flow cells that permitted direct, real-time bright-field and epifluorescence microscope observation of stained microgel particles and microorganisms in the flow stream (Fig. 1). Additionally, we could follow the development of early biofilm with bright-field microscopy on the inner surface of the top cover plate of the flow cell and with confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM) on removable silica inserts (*Materials and Methods*).

In all flow cell experiments, we consistently observed numerous particles of protobiofilm and uncolonized TEPs suspended within the seawater stream (Fig. 2 and *Movie S1*). These microgel clusters were stained with Alcian Blue and SYTO 9 and ranged from several square microns to more than 1 mm^2 in upper surface area. Staining with Alcian Blue may have caused some deformation of the microgel morphology, but this would not affect the validity of our results.

Protobiofilm particles were heavily colonized by various morphological forms of bacteria and occasionally also harbored picoeukaryotic algae, recognized by their chlorophyll *a* autofluorescence. Protobiofilm clusters such as those visualized in Fig. 2A

Fig. 1. Schematic overview of the flow-cell and experimental setup (see *Materials and Methods* for details).

Fig. 2. Bright-field and epifluorescence overlay images of in situ planktonic protobiofilm (A and C) and uncolonized TEPs (B and D) visualized in seawater passing through a flow cell. Bacteria (green) were stained with SYTO 9 and TEPs (blue) with Alcian Blue. Picophytoplankton (red) was identified by chlorophyll *a* autofluorescence.

and C, may act as planktonic hot spots of microbial metabolism. Within the confines of these heavily colonized microgel particles, diffusion of signaling molecules released by prokaryote cells should be greatly restricted, thereby enhancing the efficiency of quorum sensing and other forms of microbial communication (30).

In addition to protobiofilm, many relatively large (>10- μm) TEPs without, or with very few, associated bacteria (Fig. 2B and D) were also seen in the freshly sampled seawater flow stream. Therefore, the protobiofilm (i.e., heavily colonized TEPs) observed clearly could not have resulted from EPSs secreted by “free-living” single planktonic bacteria or by detachment from some preexisting biofilm. Rather, we posit that these protobiofilm clusters were planktonic, detrital particles already bearing microbial “passengers” or derived from precursor colloids that coalesced to form microgels (6), which were then colonized by bacteria and other microorganisms. As detailed below, the presence of large numbers of both protobiofilm particles and uncolonized TEPs in the overlying water has considerable implications for the process of aquatic biofilm formation.

Surface Conditioning by Organic Polymers and Colloids. AFM imaging and force measurements on the surfaces of the silica inserts (Fig. 1) gave descriptive and quantitative data on the initial development of a highly sticky, thin organic layer that could only have derived from organic polymers and colloidal nanogels in the seawater (4–6). This layer corresponded to the “classic” conditioning film that has been studied previously by biofilm researchers (20–22).

At 0.5 h, AFM showed extensive areas of the silica surfaces covered by a thin (~10–150 nm), very soft, and uneven film of organic material (Fig. 3A). Adhesion forces on this layer ranged between 0.83–3.58 nN. By 4 h, there was more widespread surface coverage by this organic layer (Fig. 3B) with increasing thickness (up to 250 nm) and adhesion from 8.56 to 8.99 nN. This layer appeared to be composed of an array of apparently similar, globular clusters with a variable surface topography (Fig. 3B). The data and images shown in Fig. 3 are probably characteristic of the conditioning film composed of organic polymers and colloids that forms on surfaces immediately upon exposure to seawater (24–26). A liquid cell to prevent dehydration of the surface areas was used during AFM measurements; therefore, we are reasonably confident that these results accurately reflect

We were unable to measure the adhesive forces on very prominent, organic patches that were visible in the 20 $\times$ lens of the AFM because the microscope cantilever tips became irreversibly stuck or broke when we attempted to approach these structures. Nevertheless, it was evident that these micron-thick, organic patches were extremely soft and adhesive and most likely corresponded to the Alcian Blue- and Con A-staining areas seen with bright-field microscopy and CSLM (see below).

Bright-field and epifluorescence microscope observations at 1 and 4 h showed a continuous increase in areal coverage (up to few square millimeters) of Alcian Blue-staining areas with many

These observations with bright-field and epifluorescence microscopy and CLSM extend and confirm previous studies showing that most of the EPSs appearing in early stages of aquatic biofilm formation derive from planktonic TEPs and not from EPSs secreted by bacteria that had attached initially to the surface (2, 14, 15). As noted above, during the course of all our experiments, uncolonized TEPs and protobiofilm were observed in the seawater passing through the flow cells (Fig. 2), and we infer that these were the main sources of the early biofilm structures that formed on the flow cell and silica insert surfaces during the course of these experiments.

Biofilm Development in Untreated or Filtered Seawater. To evaluate the impact of planktonic protobiofilm and TEPs on early biofilm development, we ran three flow cell experiments with either untreated seawater or seawater filtered through GF/F filters to reduce the concentrations of large microgels in the feedwater. The first two experiments focused on the initial stages of biofilm formation (up to 4 h). The third experiment (with CLSM observations only) was extended to 24 h to check for longer term differences

between biofilm developing in untreated or in filtered seawater (see *Materials and Methods*).

In all experiments, GF/F filtration effectively removed the bulk of larger sized ($>2\text{-}\mu\text{m}$) particles (89–92%), whereas most of planktonic bacteria (68–58%) remained in the filtered seawater (see *Table S1*). Although only a relatively small fraction (26–47%) of Alcian Blue-staining particles was retained by the GF/F filters, this treatment appeared to effectively remove most of the protobiofilm and larger TEPs (see below). The concentration of TEPs in the untreated seawater used in the third experiment was much lower than in the previous experiments but normal [$194 \pm 7\text{ }\mu\text{g}$ gum xanthan (GX) L^{-1}] for winter coastal waters in this area (2).

In all three experiments, in situ bright-field and epifluorescence microscopy of the feedwater revealed only occasional uncolonized TEPs and no distinct protobiofilm clusters in the filtered seawater flow, whereas both kinds of particles were present in the untreated seawater. In the third experiment, the amounts of TEPs measured in the filtered seawater flow increased significantly from 0.5 to 24 h (from 170 ± 9 to $230 \pm 23\text{ }\mu\text{g}$ GX L^{-1}). Most probably, these particles were formed abiotically from smaller-sized TEP precursors because of turbulence caused by the continuous stirring of the filtered seawater reservoir (Fig. 1) throughout the run time of the experiment (6). These newly formed TEPs were likely the source of the scattered Alcian Blue-staining areas that appeared after 4 h on the inner surfaces of flow cells with filtered seawater feed, as well as the Con A-staining structures observed with CLSM at 24 h (see below).

Bright-field and epifluorescence microscopy observations made during the first two experiments showed a drastic difference in the early development of biofilm caused by the removal of large TEPs and protobiofilm from the feedwater. By 0.5 h, there was ~ 300 -fold greater areal coverage by patches of Alcian Blue-staining material in flow cells with untreated seawater compared with those receiving filtered seawater. In both cases, we observed single bacterial cells attached to the apparently clear glass surfaces.

At 4 h, the ratio of surface areas in untreated vs. filtered seawater flow cells had decreased to ~ 7 , mostly because of an increase in coverage by adhering Alcian Blue-staining material in flow cells with filtered seawater. By this time, however, flow cells with untreated seawater showed many patches of Alcian Blue-staining areas and bacteria (similar to Fig. 3C). In marked contrast, only scattered patches of Alcian Blue-staining areas and numerous single, attached bacteria were observed on flow cells with filtered seawater.

In all three experiments, CLSM analyses also showed marked changes in the kinetics of early biofilm formation with untreated or GF/F-filtered seawater. In the third experiment, by 0.5 h, the volume of developing biofilm [microgel/EPs (Con A staining) plus bacteria (SYTO 9 staining)] in flow cells with untreated seawater was 12.8-fold of that measured in flow cells with filtered seawater (Fig. 5). However, the thickness ($28 \pm 2\text{ }\mu\text{m}$; $n = 10$) of the developing biofilm with untreated seawater was only two to three times greater than that in filtered seawater. The differences in biofilm development in untreated or filtered seawater remained very evident by 24 h (Fig. 5A). In the untreated seawater, although the silica insert surface area was still not contiguously covered, there was a relatively dense coverage of large biofilm patches (Fig. 5B), whereas only much smaller biofilm patches with sparse areal coverage were observed in filtered seawater (Fig. 5C). From 0.5 to 24 h, the volume of developing biofilm patches measured by CLSM increased in both untreated and filtered seawater about twofold (from $22,918$ to $45,449\text{ }\mu\text{m}^3$ and from $1,788$ to $4,433\text{ }\mu\text{m}^3$, respectively). However, in both cases, the measured thickness of the patches of microgel/EPs remained almost unchanged. This observation is consistent with the idea that most of the biofilm development over 24 h took the form of greater areal coverage rather than increasing thickness.

Fig. 5. (A) Changes with time in biofilm volume (measured by CLSM) in flow cells with untreated or GF/F-filtered seawater. The numbers above the filtered values show the ratio of biofilm volume measured in untreated and filtered seawater. Mean and SE were calculated from 10 to 15 randomly sampled images. (B and C) CLSM images of flow cell surfaces after 24 h with filtered (B) or untreated (C) seawater. Polysaccharides (blue) and bacteria (green) were stained with Con A and SYTO 9, respectively.

In summary, the much slower development of biofilm in GF/F filtered seawater, with depleted concentrations of protobiofilm and large TEPs, highlights the importance of these microgel particles in facilitating the initial phases of biofilm formation. These experiments also showed that even when large TEPs and protobiofilm were removed from the overlying water, early biofilm could still develop according to the classical pattern outlined in the introductory section of our article (20–22), albeit at a much slower rate.

Revised Paradigm for Aquatic Biofilm Formation. The results of this and previous studies (2, 21, 22) lead us to propose a revised paradigm for aquatic biofilm formation that takes into account the previously unrecognized role of microgel particles such as TEPs and protobiofilm in facilitating and accelerating this process (Fig. 6). We describe the early stages of biofilm development as follows:

- i) Conditioning of a pristine surface begins immediately upon contact with seawater. In addition to a thin ($<250\text{-nm}$) conditioning layer formed by organic polymers and colloids (Figs. 3 and 6A), occasional thicker ($>100\text{ }\mu\text{m}$) patches of larger microgel particles such as TEPs and protobiofilm adhere to the surface (Figs. 4 and 6B and C). These highly adhesive, carbon-rich structures alter the architecture and chemical properties of the surface, thus providing a favorable substrate for the attachment of bacteria and additional microgel particles. Single bacteria also make contact with clean surface areas, but, at first, most do not attach permanently (Fig. 6D).
- ii) During the first ~ 30 min of seawater/surface interaction, further TEPs and protobiofilm particles (Figs. 3 and 6B and C) adhere firmly to the surface. Attached protobiofilm, with its complement of fully functioning microbial communities, provides a jump-start for the early development of biofilm. During this time, single bacteria also attach irreversibly to the preconditioned surface (Fig. 6E).

Fig. 6. Schematic illustration showing the involvement of organic polymers and colloids, TEPs, and protobiofilm in the initial stages of aquatic biofilm formation. Immediately upon exposure to seawater, organic polymers and colloids (A) and microgels such as uncolonized TEPs (B) and protobiofilm (C) begin to attach to pristine surfaces. Single cells of planktonic bacteria also attach reversibly (D) or irreversibly (E) to conditioned surfaces. With time (minutes to hours), a contiguous coverage of mature biofilm (F) develops (see text for details).

iii) Under favorable environmental conditions, a widespread 3D network of early mature biofilm, derived mainly from TEPs and protobiofilm, becomes established within a few hours (Figs. 3 C and D, 5B, and 6F). Bacterial populations associated with the attached microgels and also single bacteria adhering to the surface begin to grow out and proliferate EPSs, as described by the standard model of biofilm formation (20–22). TEPs, protobiofilm, and single bacterial cells from the overlying feedwater probably continue to attach and adhere to areas already covered by developing biofilm.

Conclusions

The custom-designed flow cells used in these experiments enabled us to visualize uncolonized TEPs and protobiofilm *in situ* in the seawater feed and to follow the direct involvement of these microgel particles in biofilm development. Our results provide evidence that large Alcian Blue-staining areas initially appearing on surfaces in the flow cells could only have derived from protobiofilm and TEPs in the feedwater and were not EPSs generated by adhering, single bacteria, or bacterial aggregates. In addition, experiments comparing the initial stages of biofilm formation in filtered or in untreated seawater clearly illustrated the importance of protobiofilm and TEPs in accelerating this process.

Based on these results, we have formulated a revised paradigm in which the direct attachment of TEPs and “prefabricated biofilm” in the form of protobiofilm begins immediately upon exposure to overlying water, accelerating biofilm formation. This occurs concomitantly with the “classic” well-documented, phased process of aquatic biofilm development whereby rapid conditioning of surfaces by organic polymers and colloids (shown by AFM observations in this study) facilitates the attachment of bacterial cells and aggregates that grow out, extrude EPSs, and form mature biofilm. The model presented here implies that planktonic microgel particles are intimately involved in the initial stages of most kinds of marine and freshwater fouling. Moreover, recognition of the role of protobiofilm and planktonic microgels in aquatic biofilm formation can have applied importance for the water industry in which fouling of filtration membranes and other surfaces is a major concern.

Materials and Methods

Flow Cell Experiments. In Fig. 1, we show a schematic overview of the experimental system used in this study. Untreated coastal surface seawater, freshly collected from near surface at a station ~500 m off-shore from Hadera, Israel, was passed through a series of custom-designed flow cells (inner volume, 8 mm³) that enabled direct, real-time bright-field and epifluorescence microscope observation of particles and microorganisms in the flow stream, as well as the developing biofilm adhering to the inner surface of the top glass cover plate (Fig. 1). Further analyses by CLSM and AFM were made on biofilm that developed on removable 7-mm² silica sheets, which were attached with adhesive pads (Veeco Instruments) to the inside of the bottom plate (Fig. 1). For each experiment, we used five flow cells in parallel, held vertically (except for brief observations under the microscope) to avoid the attachment of bacteria or particles by gravity to either the inner surfaces of the flow cell cover plates or to those of the silica inserts. Seawater flow through these cells was maintained at ~8 mL min⁻¹ at room temperature (~22 °C) using a peristaltic pump located downstream from the flow cells to ensure minimal perturbation to the water current. One flow cell with sterile F/2 artificial seawater medium (34) served as a control for AFM measurements on the silica insert surfaces. A second flow cell with seawater feed was stained (see below) at 0.5, 1.0, and 4.0 h and served for real-time monitoring of biofilm development on the inner glass surface of the flow cell. At each time point, the water flow was temporarily stopped and the cell was stained for observation by bright-field and epifluorescence microscopy by injecting 300 µL of 35 nM SYTO 9 (Invitrogen) and 500 µL of Alcian Blue (Sigma; 0.4% wt/wt at pH 2.5) simultaneously through the inflow port (Fig. 1) directly into the flow cell. After 7 min, the flow was resumed and microscope images of the stained material on the inner surface of the upper cell flow cover plate were immediately taken as described below. The three remaining flow cells were sampled at 0.5, 1, and 4 h, respectively; at these times, the silica inserts were removed and processed for examination by CLSM and AFM (see below).

Biofilm Development in Untreated or Filtered Seawater. To examine the specific contribution of TEPs and protobiofilm to biofilm formation, we ran a series of three flow cell experiments using seawater that was either untreated or filtered through a GF/F filter (~0.7- μm cutoff) to remove the larger microgel fraction while retaining most of the free planktonic bacteria. Samples for TEP concentration, total >2- μm particle count, and bacterial abundance were taken from the untreated and filtered seawater before each experiment (Table S1). Untreated or GF/F-filtered seawater was passed in parallel through four paired sets of flow cells. In the first two experiments, 1 pair of flow cells was stained at 0.5, 1 and 4 h with Alcian Blue and SYTO 9 for observation of TEPs and protobiofilm in the seawater flows and on the inner surface of the upper cell flow cover plate by bright-field and epifluorescence microscopy as described above. At each of these time points, the flow to one pair of flow cells was stopped and the silica inserts were stained and examined by CLSM. In the third experiment, only three sets of paired flow cells with silica inserts were used. These were sampled at 0.5, 4, and 24 h; at these times the silica inserts were removed, stained and examined with CLSM (see below).

Real-Time Light and Epifluorescence Microscope Imaging. During the experimental runs, immediately upon staining with Alcian Blue and SYTO 9 (see above), we captured images of TEPs and planktonic bacteria in the flow stream at 5-s intervals ([Movie S1](#)) using a bright-field/epifluorescence microscope (Nikon; Eclipse 80i) with a far focal field lens (Nikon; plan fluor 20×/0.45). By using another lens (Nikon; plan fluor 40×/0.75), we were also able to examine Alcian Blue-staining material and bacteria adhering to the inner top cover surfaces of the flow cells. All bright-field and epifluorescent image stacking and analysis was done using Image J software (<http://rsbweb.nih.gov>).

CLSM. At each sampling time, silica inserts were removed from the flow cells and placed in Petri dishes with sterile F/2 medium. Each silica sheet was then gently rinsed with F/2 medium to remove loosely adhering bacteria and other microorganisms and stained with 0.5 nM SYTO 9 and with 55 μ M Con A (Alexa Fluor 647; Invitrogen) for 7 min⁻¹ in the dark. The samples were scanned with a CLSM (Leica) equipped with a submerged lens (400x) for in situ observations. For further method details, see [SI Materials and Methods](#).

AFM. AFM observations were made with an ICON Atomic Force Microscope (Bruker). To mimic natural conditions and to prevent biofilm dehydration, 7-mm² sections of rinsed silica inserts were analyzed in a liquid cell filled completely with an aqueous solution of buffered F/2 medium (pH 8.2). All tips (NP-5; Digital Instruments) were treated in a UV/oxygen cleaner before use. Surface architecture was imaged using tapping mode (drive frequency

and amplitude, ~29 kHz and 550 mV, respectively) to minimize contact of the tip with the film. For force measurements, the rate was set at 0.5 Hz with image resolution at 512 samples/line. For further method details, see [SI Materials and Methods](#).

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