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UNIVERSITY OF CALIFORNIA SAN DIEGO

**Underwater Microscopic Imaging & Velocimetry
for *In Situ* Studies of Benthic Marine Environments**

A dissertation submitted in partial satisfaction of the requirements for the degree of
Doctor of Philosophy

in

Electrical Engineering
(Applied Ocean Science)

by

Andrew David Mullen

Committee in charge:

Jules S. Jaffe, Chair
Sadik C. Esener, Co-Chair
William S. Hodgkiss
Yu-Hwa Lo
Eugene R. Pawlak

2018

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The Dissertation of Andrew David Mullen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego
2018

DEDICATION

In recognition of their encouragement, love, and support, this dissertation is dedicated to my parents John and Lori Mullen, and my sister Morgan Spenla.

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Chapter 2, in full, is a reprint of the material as it appears in Nature Communications 2017: Mullen, Andrew D; Treibitz, Tali; Roberts, Paul L.D.; Kelly, Emily L.A.; Horwitz, Rael; Smith, Jennifer E.; Jaffe, Jules S., Nature Publishing Group, 2016. The dissertation author was a joint primary investigator and joint primary author of this paper.

Chapter 3, in full, is material currently being prepared for publication: A.D. Mullen, P.L.D. Roberts, A. DePavia, A. Genin, J.S. Jaffe “Underwater Micro-Particle Tracking Velocimetry for *In Situ* Observation of Microscopic Flows Over Benthic Organisms.” The dissertation author was the primary investigator and author of this material.

Chapter 4, in part, is currently being prepared for publication: A.D. Mullen, A. Genin, P.L.D. Roberts, J.S. Jaffe “*In Situ* Micro-Scale Fluid Dynamics at the Coral-Ocean Interface.” The dissertation author was the primary investigator and author of this material.

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A.D. Mullen, T. Treibitz, P.L.D. Roberts, E.L.A. Kelly, R. Horwitz, J.E. Smith, J. S. Jaffe, “Underwater Microscopy for *In Situ* Studies of Benthic Ecosystems.” Nature Communications (2016).

A.D. Mullen, T. Treibitz, P.L.D. Roberts, J.S. Jaffe, “An Underwater Microscope for *In Situ* Imaging of Seafloor Organism.” Optical Society of America, Conference on Novel Techniques in Microscopy (2017).

ABSTRACT OF THE DISSERTATION

Underwater Microscopic Imaging & Velocimetry
for *In Situ* Studies of Benthic Marine Environments

by

Andrew David Mullen

Doctor of Philosophy in Electrical Engineering
(Applied Ocean Science)

University of California San Diego, 2018

Jules S. Jaffe, Chair
Sadik C. Esener, Co-Chair

Many fundamental biological and physical processes taking place in benthic marine environments such as coral reefs, kelp forests, and sea grass beds occur at scales of a millimeter or less. For example, large reef ecosystems are cumulatively built by many coral colonies, these colonies are in turn composed of numerous individual coral polyps each on the order of one millimeter in diameter. The polyps feed on micro-scale plankton and get photosynthetic energy from single-celled symbiotic algae. Furthermore, micro-scale fluid motions and mixing surrounding coral polyps impact their ability to exchange critical dissolved solutes such as oxygen and nutrients with the surrounding water. Natural ocean environments are also complex

and dynamic systems that are difficult or impossible to fully replicate in the lab. A distinct need thus exists for direct observations of important marine processes *in situ*. This thesis describes the development of novel underwater microscopic imaging and velocity measurement techniques for studying micro-scale biological and physical processes at the seafloor in the natural environment. These techniques are then applied specifically to study reef-building corals.

First, I describe the development and application of the Benthic Underwater Microscope (BUM). This diver-operated instrument performs non-invasive imaging of subjects on the seafloor *in situ* at nearly micrometer resolution. The instrument's optical system combines a long working distance microscopic objective lens, a flexible electronically tunable lens, and focused reflectance illumination. The BUM was deployed in the ocean to study coordinated coral polyp behaviors, interspecies coral polyp competition, and algal colonization of bleached coral colonies.

Second, I modify the BUM to measure two-dimensional fluid velocities at micro-scales using micro-particle tracking velocimetry (μ PTV). This is achieved by incorporating dark field illumination, rapid image pair acquisition, active particle seeding, and application of particle tracking algorithms. The system was used to measure fluid velocity fields at sub-millimeter scales around coral polyps in the ocean, and a viscous boundary layer was observed directly at the coral's surface.

Finally, the underwater μ PTV instrument was used to study spatiotemporal dynamics of fluid motion around individual coral polyps *in situ*. We measure shear stress at the coral's surface and show waves periodically modulating the near-surface flow environment. The tools developed here provide a means to bring the lab into the ocean, offering a new window into important marine processes.

Chapter 1

Introduction

1.1 Benthic marine environments & micro-scale processes

1.1.1 Benthic environments

The ocean can broadly be divided into two regions: benthic environments on or near the seafloor, and pelagic environments in the water column. Benthic environments include diverse coastal ecosystems such as coral reefs, sea grass beds, kelp forests, rocky reefs, and mangroves. The ocean benthos also includes deep-sea habitats such as abyssal planes, methane seeps, and hydrothermal vents. Benthic ecosystems have high economic and biological value. They support commercial fisheries; act as breeding grounds and nurseries for juvenile fish; drive tourism industries; provide coastal protection from erosion and storms; and impact biogeochemical cycles including carbon sequestration. Coral reefs specifically have an estimated global economic value of approximately US\$ 30 billion per year¹. Corals also host approximately 25% of the ocean's fish biodiversity, while occupying less than one tenth of one percent of the seafloor².

1.1.2 Micro-scale biological processes & coral reefs

A variety of micro-scale processes (those occurring at scales of less than a millimeter) significantly impact the health of these benthic ecosystems. As one example, coral reefs form large physical structures that can extend for 10s of kilometers and create complex habitats that support diverse marine life. The reef structure itself is collectively built by many coral colonies, and each colony in turn consists of hundreds to millions of small coral polyps on the order of

1 mm in diameter each (Fig. 1.1). The coral polyps lay down an external calcium-carbonate skeleton, which builds the coral colony and physical reef over time. As compared to the terrestrial world, the analogy can be made that “corals are to coral reefs as trees are to forests”³.

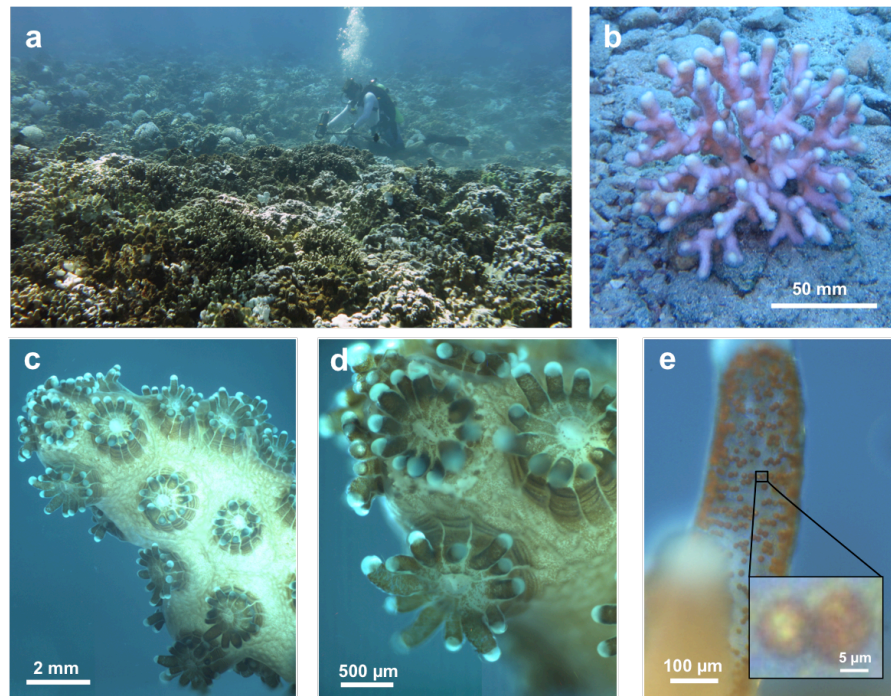


Figure 1.1 | Scale of the coral reef, coral colony, and coral polyp. (a) Coral reef composed of many individual coral colonies (scientific diver in background for scale). (b) A small branching coral colony (~100 cm diameter) on the seafloor. (c) A branch of the coral *Pocillopora damicornis*. The branch features many polyps, with the individual polyps sharing a connective tissue known as the coenosarc. Image taken in lab with prototype of Benthic Underwater Microscope (BUM). (d) Several individual coral polyps (~1 mm diameter). Image taken in lab with prototype of the BUM. (e) Tentacle of polyp with inset showing zooxanthellae (~10µm diameter) inside coral tissue. Image taken in the lab.

Each coral polyp is itself a small marine invertebrate animal that conducts a variety of important micro-scale activities. They obtain the energy to build reefs largely through a symbiotic relationship in which they host photosynthetic single-celled algae (~10 µm in diameter), known as zooxanthellae⁴. Coral polyps additionally prey on plankton in the water column⁵. Micro-scale biological processes of interest on coral reefs include: coral bleaching of

their single-celled symbiotic algae^{4,6}, rates of coral predation on plankton⁷, competition between different coral species⁸, competition dynamics between corals and turf algae^{9,10}, the settlement and attachment of coral larvae^{11,12}, the propagation of coral diseases¹³, and coordinated behavior between coral polyps.

Micro-scale processes impacting the health of individual coral polyps ultimately affect the health of coral colonies, and can drive large-scale changes to the entire reef ecosystem³. It is thus important to observe and study corals at the scale of the individual polyp and the microenvironment the polyp experiences. This perspective can be described as the “coral polyp’s eye view” of the world, similar to the “plant’s eye view” concept used in terrestrial ecology¹⁴.

The challenge of observing and connecting processes across a wide range of scales is of central importance to the study of many marine ecosystems¹⁵. Calls for technology to perform micro-scale imaging in the ocean include that of V. Smetacek who asked, “Could such an instrument (an *in situ* computerized telemicroscope) do for microbial ecology what Galileo’s telescope did for astronomy?”¹⁶.

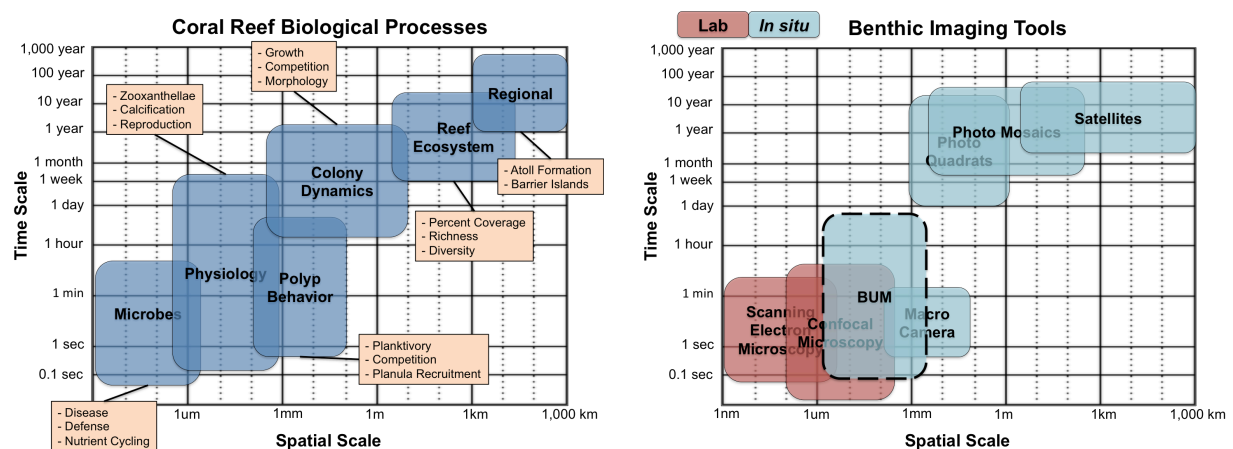


Figure 1.2 | Scales of biological processes occurring on a reef. (a) Log-log plot of biological processes on reef environments. **(b)** Log-log plot of available imaging technologies. In many cases processes at small time and space scales drive changes to the larger ecosystem.

1.1.3 Micro-scale physical processes

In addition to observations of benthic organisms themselves, the physical micro-environment they experience is also of significant importance¹⁷. Specifically, fluid boundary layers have been referred to as the “most pervasive physical influence on the biology of many aquatic organisms” due to their impact on mass and momentum transfer¹⁸. In order to maintain basic physiological functions (such as respiration, photosynthesis, and calcification), benthic organisms need to both uptake a variety of dissolved solutes from the surrounding water and eliminate waste products¹⁹. Important exchanges with the surrounding water include the transfer of nutrients, oxygen, carbon dioxide, calcium, and reactive oxygen species (Fig. 1.3).

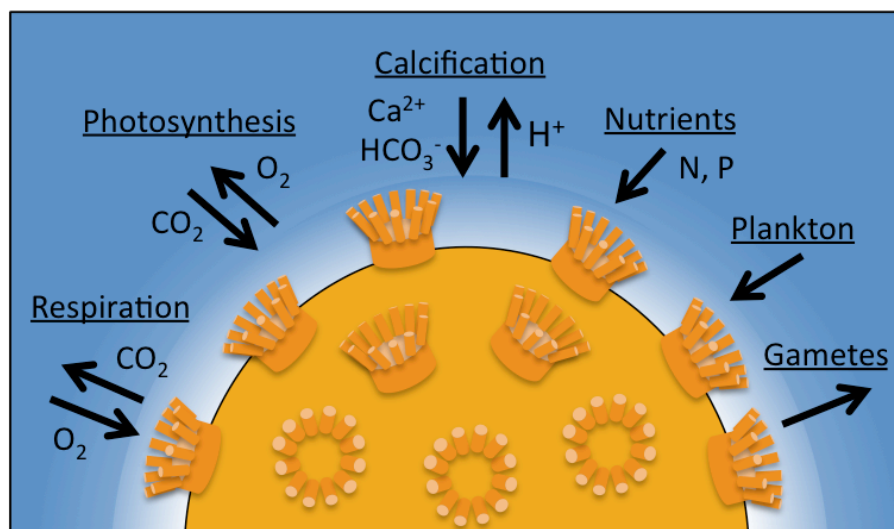


Figure 1.3 | Coral mass exchanges. Biological processes conducted by corals, and the exchanges of dissolved solutes and other materials needed to support these processes.

Fluid motions slow at physical boundaries. This reduces mixing and at small scales leads to the formation of a fluid dynamic viscous boundary layer (VBL), typically 1-10 mm thick at the ocean benthos. Within the VBL a chemical diffusion boundary layer (DBL) forms, typically 100-1,000 μm thick above benthic organisms and surfaces (described in more detail in Section

1.3). The DBL slows the transport of dissolved gasses and nutrients to and from the benthos. This reduces mass exchange and may limit basic metabolic functions of marine organisms; potentially impacting diverse subjects such as corals²⁰⁻²², kelp²³⁻²⁵, and sea grasses²⁶, as well as microbial mats and bacteria in sediments²⁷⁻²⁹. Significantly, the DBL thickness is controlled by fluid dynamics at the surface-water interface. Fluid motions in this region additionally have important impacts on biological larval recruitment^{11,12,30}, filter feeding and prey capture^{7,31}, and bio-driven mixing currents^{32,33}.

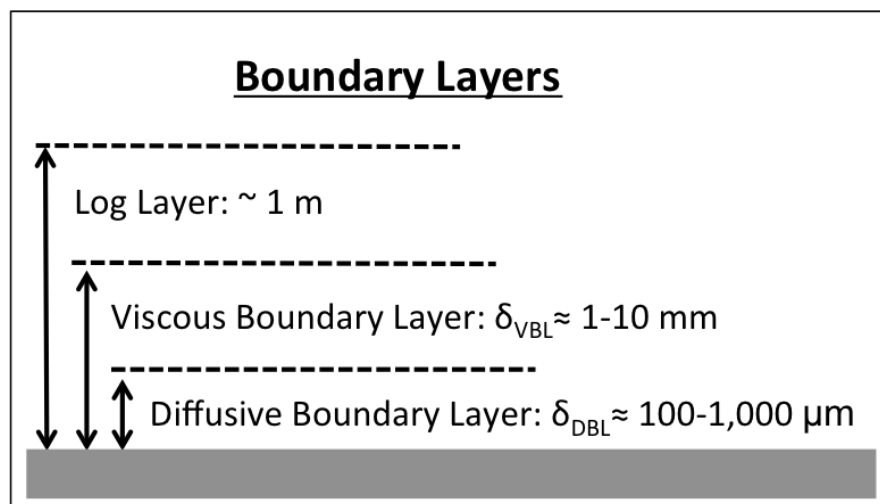


Figure 1.4 | Schematic of boundary layers at the surfaces of benthic organisms and sediments. Near surfaces turbulent mixing is reduced. Within the VBL the transfer of fluid momentum is dominated by viscosity. Within the DBL the transfer of dissolved materials is dominated by molecular diffusion.

1.1.4 Relevance of micro-scale processes to coral stressors

Coral reefs are currently experiencing widespread declines in health^{34,35}. This is being driven by the effects of climate change including ocean warming and acidification; as well as local anthropogenic stressors including overfishing and excess nutrient inputs. Understanding the specific mechanisms by which environmental changes impact the health of corals is important

for determining the best measures to protect and conserve these ecosystems.

One example of a major consequence of ocean warming is coral bleaching^{3,4,34}. This occurs when coral polyps eject their symbiotic zooxanthellae, a process known as bleaching, due to excess temperature and high light conditions^{4,6}. Bleaching severely weakens corals and frequently leads to widespread mortality³⁴. Another driver of coral declines is turf algal overgrowth of reefs^{9,36}. Degraded reefs dominated by algae are said to have undergone a ‘phase shift’³⁵. Both coral bleaching and algal overgrowth can dramatically impact reef ecosystem health, but significantly these processes occur at micro-scales across the reef. Additionally, the flow environment over corals has also been shown to have important impacts on the ability of corals to cope with stressors such as ocean warming³⁷ and acidification³⁸.

1.1.5 *In situ* observational need

Shallow oceans are complex physical and biological environments. Waves, currents, and eddies interact across a range of scales to control water motions^{17,39}. Important physical conditions including temperature⁴⁰, pH⁴¹, and irradiance fluctuate with time and space. At the same time, organisms on the reef have complex and diverse biological interactions with each other. It is therefore difficult or impossible to fully replicate the intricacies of these natural systems in the lab. A need thus exists for direct, *in situ* observations of important environmental processes under natural conditions. This is true at a wide range of scales, including at the micro-scale. In order to provide these *in situ* micro-scale observations traditional sampling approaches must be flipped – rather than bring ocean subjects to lab, the lab must be brought into the ocean.

1.2 Underwater imaging & microscopy

1.2.1 Underwater imaging history

Early underwater imaging in the ocean dates back as far as the mid-1800s⁴². However it was not until the 1970s that quantitative scientific imaging of benthic underwater habitats was adopted by ecologists, who began performing imaging surveys of coastal environments using camera photo-quadrats and transects⁴³. During the same time period, towed platforms for imaging deep-sea environments were developed and contributed to the discovery of hydrothermal vents on the ocean floor. Today, photomosaic maps of benthic habitats over 10s to 100s of square meters are routinely collected in a variety of environments by divers and autonomous vehicles^{44,45}.

Small-scale microscopy observations have long been used to study marine specimen in the lab, including early investigations of coral behavior⁴⁶. Underwater small-scale imaging was pioneered in the 1980s by Ortner and Edgerton, who performed direct *in situ* imaging of small plankton in the water column⁴⁷ (and also imaged zooplankton recently captured from net tows⁴⁸). Since then, a wide variety of *in situ* imaging systems have been developed to image plankton in the ocean. These devices have observed a range of different size classes using a variety of imaging modalities including: shadowgraph imaging, line scan imaging, holography, free space dark field microscopy, and flow cytometry^{49,50}.

In contrast to this proliferation of *in situ* plankton imaging systems, tools for underwater microscopic imaging of benthic environments have been lacking. Previous tools for *in situ* observations of small benthic subjects have been relatively simple and invasive. These have included: lenses designed for attachment to a dive mask⁵¹, magnifying glasses⁵², drop cameras for imaging benthic sediment grain size⁵³, underwater macro-photography^{5,54}, and even a

traditional bench top microscope enclosed in underwater housing⁵⁵. The lack of tools capable of non-invasively imaging benthic subjects at high resolution has hindered efforts to connect lab studies to natural environments, and to investigate the mechanisms by which micro-scale processes impact large-scale ecosystem changes. Performing non-invasive microscopic imaging of benthic subjects at the seafloor presents a variety of challenges including the need for: active instrument positioning and focusing, reflectance illumination, a long working distance, and fast exposures in an unstable setting.

1.2.2 Microscopy history and advancements

Light microscopy has played a central role in diverse scientific fields. Throughout its history technical advances in microscopy have allowed observations of subjects 1) at increasingly high resolution and 2) under increasingly natural conditions. Such technological advances have in turn delivered important new scientific capabilities enabling novel discoveries^{56,57}. Simple optical microscopes were used by Hooke and Leeuwenhoek in the 1660s and 70s to make the first observations of microorganisms⁵⁸. Theoretical descriptions of optical image formation and the resolution limit of light microscopy were developed by Ernst Abbe and described in 1873⁵⁹, providing a foundation for modern microscopy advancements. The 20th and early 21st centuries then saw rapid growth of many advanced microscopy techniques providing enhanced contrast and resolution such as: phase contrast microscopy, confocal microscopy, super resolution imaging⁵⁷, and computational imaging⁶⁰.

Concurrent efforts have also been made to observe subjects under increasingly natural conditions. Traditional microscopy is typically conducted *in vitro*, or “within the glass”. In this case observations are conducted in a controlled environment outside of a living organism, such

as cells cultures in petri dishes. Significant research efforts have focused on developing techniques to collect microscopic observations *in vivo*, or “within the living”. In this case observations are performed on whole living organisms or tissues. *In vivo* imaging techniques are being applied to topics such as imaging of brain activity^{61,62}, pathology to detect diseases inside live organisms⁶³, and observations of active processes in live marine specimen such as coral polyps held in small fluidic chambers^{64,65}. Another important frontier (that builds on *in vitro* and *in vivo* imaging) is microscopic imaging outside of traditional lab setting – this can broadly be referred to as portable microscopy. As one example, a number of research groups are working to transform cell phones cameras into low cost point-of-care diagnostic tools for disease detection in both the developed and developing worlds^{66,67}. Compact microscopic imaging is also being developed for spacecraft as a potential tool for exploration and life detection⁶⁸. Here we are interested in using microscopic imaging to study environmental processes as they naturally occur on site, or “*in situ*”. This *in situ* environmental microscopy is especially relevant to benthic marine environments such as coral reefs and kelp forests where, as previously described: 1) large-scale ecosystem function and health is often driven by micro-scale processes and 2) the environmental complexity cannot be fully replicated in the lab.

1.2.3 Microscopy principles

Traditional light imaging systems generally share the same primary elements: (i) an illumination source emitting light, (ii) a subject with which the light interacts, (iii) a lens that collects the light and forms an image, (iv) a two-dimensional sensor that records the image. In the case of human vision the source is typically the sun, the subject is any object being looked at, the image-forming lens is the cornea of the eye, and the sensor is the eye’s retina. A traditional

light microscope typically shares these same basic components, however it is designed to resolve very small subjects. Obtaining high-resolution images requires an optical system with: high magnification, high resolving power, low aberrations, and sufficient spatial sampling by the recording medium. Additionally such a system must have proper focusing and illumination.

Resolution can be defined as the closest distance at which two separate lines or points on subject can be differentiated. The maximum optical resolving power of a conventional microscope is determined by the Numerical Aperture (NA) of its objective lens (Fig. 1.5). The NA describes the range of angles over which the microscope lens collects light:

$$NA = n \sin \theta,$$

The resolution limit based on the Raleigh criterion can then be expressed as:

$$R = 0.61 \frac{\lambda}{NA}$$

(Where n is the index of refraction of the medium in which the lens is immersed, θ is the half-angle of the maximum cone of light that can enter the lens, and λ is the wavelength of light.)

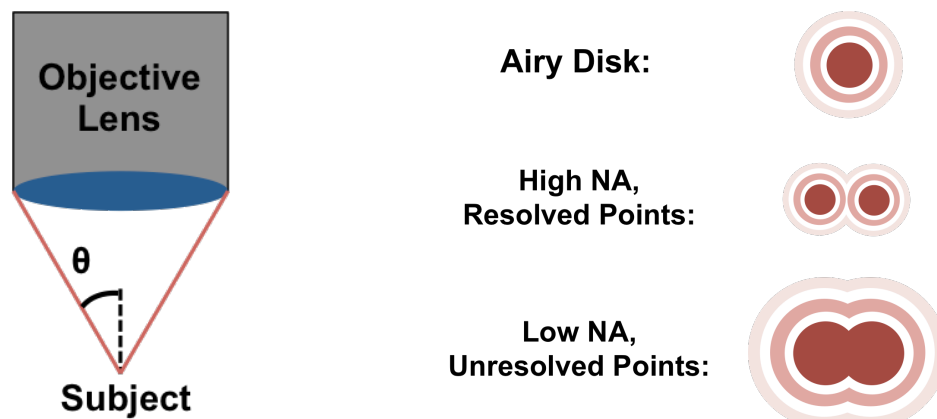


Figure 1.5 | Objective lens & airy disk schematic. (a) The NA of the objective lens is determined by the maximum half angle, θ , over which it collects light. (b) An objective lens with a large θ will have a large NA and produce small Airy Disks. This will allow the system to resolve small features. Alternatively a small θ will result in large Airy disks and low resolving power.

A short explanation for this resolution limit is that a point source of light captured by the microscope objective lens is projected onto the imaging sensor as a finite-diameter spot known as an Airy Disk (Fig. 1.5). As the NA of the objective lens increases the diameter of the Airy Disk decreases, improving the ability to differentiate or resolve small features.

A more detailed description of the imaging processes is that light is diffracted as it passes through the imaged subject, with higher spatial frequency information being diffracted at larger angles. The microscope objective lens captures light from the subject over a finite range of angles, as defined by the NA, and this light is focused at the imaging plane where it interferes to form an image. Ernst Abbe succinctly described the resulting microscope image as “the interference effect of a diffraction phenomenon”. A larger NA captures more diffraction orders allowing it to resolve smaller features. From a Fourier optics perspective, the objective lens can be described as a low pass spatial filter, with the NA defining its cutoff frequency.

An imaging system is considered to be diffraction limited if it records the maximum resolution possible for its given NA. In order to be diffraction limited: 1) the optical elements must have low aberrations, and 2) the camera sensor must sample the image projected on its surface at the Nyquist frequency (twice the maximum optical resolution) or greater.

In addition to the resolving power, other parameters of importance to the design of a microscope include the depth-of-field, working distance, and field-of-view. In conventional light microscopy a higher NA produces a thinner depth-of-field (DOF, the range over which an object is in focus). As a result, optical microscopes typically require precise focusing. The DOF follows the relationship:

$$\text{DOF} = \frac{\lambda \sqrt{n^2 - \text{NA}^2}}{\text{NA}^2}.$$

The working distance describes the space between the in-focus plane and the objective lens. For *in situ* microscopy we desire a long working distance so as not to disturb the underwater subject being observed. Achieving a long working distance with a high NA requires a large diameter objective lens. As the size of a lens increases, it becomes increasingly difficult to fabricate. However in the last few decades objective lenses (with relatively modest magnifications) have become available with working distances of 10s of millimeters. The development of such lenses has largely been driven by machine vision and automated inspection needs.

In this thesis, a long working distance objective lens in an underwater housing is used to image subjects in the ocean. As light propagates through water it may become absorbed or scattered. This presents a variety of challenges for long-range large-scale imaging systems⁶⁹, however in the work presented here the optical path is very short and as such imaging through water was not a major constraint. Light will refract as it passes from the water to through a clear port into the housing⁷⁰, this increases the working distance of the objective lens while the NA is conserved. Refraction at the port may also introduce aberrations, here these were not prohibitive due to the system's modest NA.

1.3 Boundary layer fluid dynamics & velocity measurements

1.3.1 Small-scale benthic fluid dynamics

As water flows over seafloor organisms and sediments it is slowed by friction leading to the formation of a benthic boundary layer (BBL)⁷¹⁻⁷⁵. Fluid motions here control mass and momentum flux between benthic subjects and the surrounding water, and are thus important to diverse biological and environmental processes. Small-scale water motions at distances of

millimeters or less from surface-water interfaces are particularly important, but difficult to study.

In most of the water column fluids are well mixed locally by turbulent motions. However, near surfaces fluid motions slow and viscosity causes turbulent mixing to be reduced. At typical distances of 1-10 mm from benthic subjects, turbulent mixing is sufficiently reduced such that the transfer of fluid momentum is dominated by viscous forces (Fig. 1.4, 1.6). This region is referred to as the viscous boundary layer (VBL)^{21,71,76,77}. Here fluid velocities increase linearly away from the surface, and this gradient may be used as a direct measure of wall shear stress⁷⁸⁻⁸⁰:

$$\tau_w = \mu \left. \frac{\partial \bar{u}}{\partial y} \right|_{y=0} \approx \mu \left. \frac{d\bar{u}}{dy} \right|_{y=0}$$

(Where $\mu = \nu \cdot \rho$ is the dynamic viscosity of the fluid, u is velocity parallel to the wall, and y is distance from the wall). And the friction velocity can then be calculated as:

$$u_* = \sqrt{\tau_w / \rho}$$

For turbulent flows over a flat plate the average velocity profile above the surface can be described by the ‘law of the wall’ boundary layer theory⁸¹, which defines a universal average velocity profile based on non-dimensional wall units:

$$y^+ = \frac{y u_*}{\nu},$$

and non-dimensional velocity:

$$u^+ = \frac{\bar{u}}{u_*}.$$

The theoretical ‘law of the wall’ predicts that for $y^+ < 5$ viscous forces dominate momentum transfer and velocities increase linearly from the wall (the VBL), and for $y^+ > 30$ momentum transfer is dominated by turbulent mixing and velocities increase logarithmically with distance from the wall (shown in Fig. 1.6).

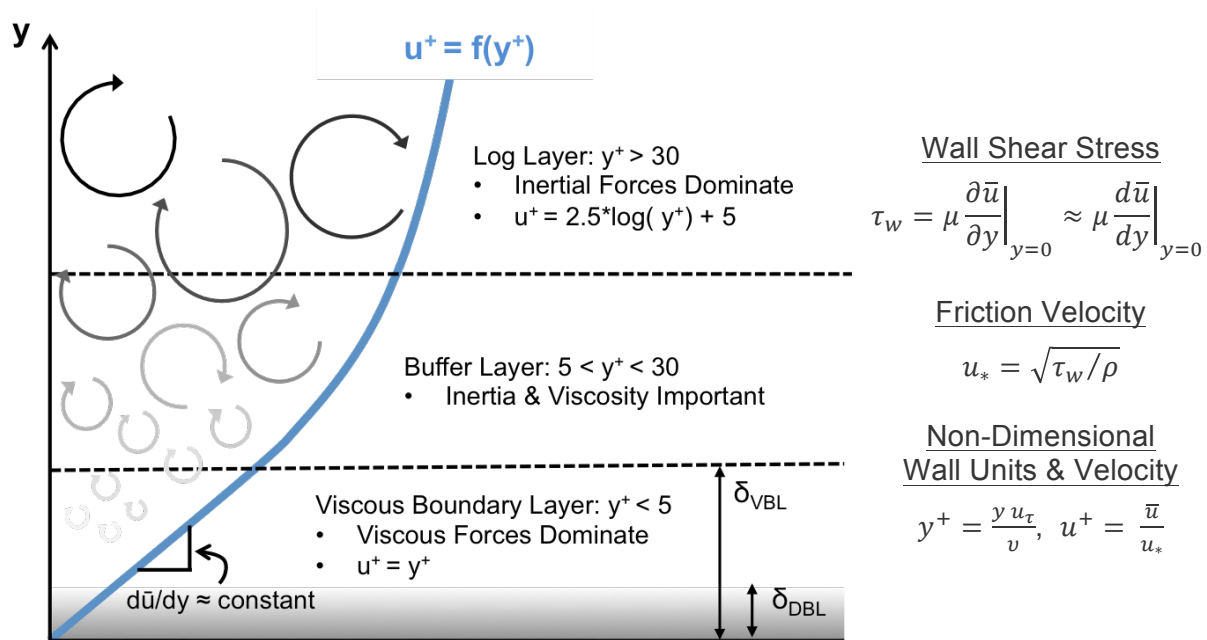


Figure 1.6 | Law of the wall velocity profile. Near the wall ($y^+ < 5$) turbulent motions are very small and viscous forces dominate momentum transfer. Further away from the wall ($y^+ > 30$) turbulent forces dominate momentum transfer. A buffer layer exists between the two regions, here both inertial and viscous forces are important to momentum transfer. In the viscous region the velocity profile is approximately linear and this gradient provides a direct measure of wall shear stress.

1.3.2 The diffusive boundary layer

At small scales within the VBL, typically 100 to 1,000 μm from benthic surfaces in the ocean, fluid mixing is sufficiently reduced such that the transfer of dissolved solutes and gasses is dominated by molecular diffusion rather than turbulent mixing. This creates a distinct chemical microenvironment known as the diffusive boundary layer (DBL)^{19,20,27}. The chemical DBL is 'submerged' within the hydrodynamic VBL^{82,83}. Within this DBL region there is a linear gradient in the concentration of dissolved solutes such as oxygen. Mass transfer through this DBL region is governed by Fick's first law of diffusion:

$$J = D \frac{\partial C}{\partial y} \approx \frac{C_{\infty} - C_0}{\delta_{\text{DBL}}}$$

Where $C_{\infty} - C_0$ is the concentration difference between the benthic surface and surrounding water, D is the coefficient of molecular diffusion, and δ_{DBL} is the DBL thickness^{72,84}.

Benthic organisms like corals need to exchange gases such as oxygen with the surrounding water in order to sustain basic physiological processes. The DBL blankets the benthos, and may act as a 'bottle neck' impeding mass transfer and potentially limiting basic metabolic activities. Significantly, thickness of the DBL is controlled hydrodynamically – higher fluid velocities increase mixing and result in a thinner DBL. The DBL thickness can be scaled from fluid velocity measurements of VBL thickness or shear stress^{82,83,85,86}. This scaling is performed using the Schmidt number, which is a ratio of the diffusion of momentum (kinematic viscosity) to molecular diffusion, ($Sc = \frac{\nu}{D}$, ν is kinematic viscosity and D is molecular diffusivity)⁸⁵.

Dissolved gases and nutrients in water typically have Schmidt numbers ranging from approximately 500 to 1,000^{19,85}. For steady flow over a flat surface mass transfer theory predicts:

$\frac{\delta_{\text{VBL}}}{\delta_{\text{DBL}}} = \text{Sc}^{\frac{1}{3}}$, this indicates that the DBL is expected to be approximately one-tenth the VBL

thickness for Schmidt numbers of interest here ($\frac{\delta_{\text{VBL}}}{\delta_{\text{DBL}}} \approx 10$)^{19,85}. This relationship can also be

used to express DBL thickness directly as a function of friction velocity: $\delta_{\text{DBL}} = \delta_{\text{VBL}} \text{Sc}^{-\frac{1}{3}} =$

$10 \frac{\nu}{u_*} \text{Sc}^{-\frac{1}{3}}$. Experimental lab measurements of steady unidirectional flow over sediments have

shown this relationship to hold true, although the leading coefficient of 10 may vary^{82,83}.

In the ocean many surfaces have complex three-dimensional structure and flows may be non-stationary – both these factors may impact the ideal scaling relationship between the DBL, VBL, and friction velocity. Direct numerical simulations of oscillating flow over coral surfaces found that this scaling relationship was more complex, however this study still concluded that “local shear stress appears to be a proxy for local mass transfer”⁸⁶. When considering the effects of oscillating flows on mass transfer it may be important to take into account the rate at which surrounding flow speeds change and the time scales for the DBL to fully develop^{84,87,88}. A time scale for the development of a DBL can be estimated based on the mean diffusion time through the DBL ($t \approx \delta_{\text{DBL}}^2 / D$), this is typically expected to be 10s of seconds to minutes based on the DBL thickness^{23,84,88}. If flows speeds in the surrounding water change faster than the diffusional time scales, then the DBL may not have time to fully develop. Additionally, surface renewal events caused by turbulent motions may quasi-periodically sweep the DBL away⁸⁹⁻⁹¹. The three-dimensional surface structure may also impact mass transfer^{84,92,93}; with more complex surface structure resulting in greater mass transfer, and faster flow rates causing the DBL to more closely follow the surface. Finally, in some cases horizontal advection may also play an important role⁸⁴.

1.3.3 Micro-scale chemical measurements

The DBL chemical micro-environment at the surface of benthic organisms and sediments can be studied using needle tipped micro-electrodes^{29,94,95}. These provide a point measurement of dissolved solute concentration and can be designed to detect a variety of different chemical species. Using micro-manipulators these sensors can record vertical profiles with spatial accuracy on the order of micrometers to tens of micrometers⁹⁵. *In situ* microsensor studies have demonstrated the existence and importance of the DBL over sediments in the ocean^{28,29,96}. Micro-electrode work in the lab has also shown that the DBL plays an important role for corals in photosynthesis and respiration^{20,97}, calcification^{38,98}, and competition with algae^{99,100}. Microsensors have shown the DBL thickness varies inversely with fluid velocities^{20,28,84} and is influenced by the small-scale surface topography^{92,93} including changes over coral polyps²¹.

However micro-sensors have some significant limitations. They only provide a point measurement, and while they can be profiled it is difficult to study spatiotemporal processes. They are inherently invasive and their structure may impact the measured DBL thickness¹⁰¹. Additionally, *in situ* micro-sensors applications have mostly been used to study benthic sediments – there have been very limited *in situ* investigations of corals^{100,102} or other spatially complex subjects. Finally, they only measure the chemical environment and not fluid motions, which ultimately drive the system.

1.3.4 Micro-scale fluid measurements

There have been very few *in situ* measurements of fluid velocities with sufficient spatial resolution to observe the VBL at the ocean benthos. In the 1970's an experiment using profiling hot wire anemometry directly measured a linear velocity profile in the VBL overlying seafloor

sediments^{76,103}. Much later, a 2014 study used a profiling acoustic Doppler velocimeter (ADV) to similarly observe the VBL over marine sediments and stated that “in marine environments, the viscous sublayer has been observed only once before”⁷⁷. ADV and hot wire anemometry techniques both provide only one-dimensional velocity profiles and may be difficult or impossible to apply over spatially complex organisms *in situ*. Additionally, profiling ADV measurements must be interpreted with caution as they may experience significant noise within 2-5 mm of surfaces and provide only 1 mm resolution^{104,105}. While anemometry profiles are very slow and only provide absolute velocity magnitude.

Another approach to measuring fluid velocities is image velocimetry¹⁰⁶. This technique uses the displacement of tracer particles between two successive images to calculate quantitative two-dimensional fluid velocity fields. There are two general types of image velocimetry: particle image velocimetry (PIV) which tracks the movement of groups of particles between images using cross-correlations, and particle tracking velocimetry (PTV) which tracks the displacement of individual particles between images. PIV and PTV are inherently non-invasive, provide whole field two-dimensional observations, and offer robust direct measurements of fluid motion.

A variety of macro-scale underwater PIV systems have been developed to investigate pelagic and benthic aquatic environments. Such systems have been used to investigate bottom turbulence¹⁰⁷⁻¹⁰⁹, flow over corals^{32,110}, jelly fish swimming¹¹¹, and a variety of additional fluid dynamic topics. These instruments have conducted measurements at distances of several millimeters from benthic surface, with fields-of-view ranging from approximately 0.5m x 0.5m to 2.5cm x 2.5cm (and corresponding spatial resolutions of approximately 6.0 mm to 0.6 mm)^{97,112}. Underwater holography has also been used to perform small-scale PIV and tracking of

plankton in the water column¹¹³. However no image velocimetry system has reported direct *in situ* observations of the VBL at the ocean floor.

In the lab μ PIV and μ PTV have been used to measure micro-scale water motions. This technique was originally developed to study flows in microfluidic devices¹¹⁴, and has been applied to topics such as measuring wall shear stress in veins *in vivo*¹¹⁵⁻¹¹⁷. Recently, lab based μ PIV has been applied to study water motions around small marine organisms, observations have included cilia driven currents around coral polyps³³ and starfish¹¹⁸, as well as the swimming patterns of plankton^{119,120}. However these studies have typically been conducted in small millifluidic chambers, which prohibit observations of interactions with large-scale flows and natural ocean water motions. Another approach, long distance μ PTV was recently developed to non-invasively measure micro-scale fluid motions over surfaces in turbulent flows^{79,121,122}. This is achieved using long working distance microscopic objective lenses and the method provides measurements of wall shear stress. Long distance μ PTV has been applied to mechanical objects such as structured surfaces^{123,124}, flat plates⁸⁰, engines^{125,126}, and helicopter blades¹²⁷.

1.4 Thesis outline

This thesis seeks to develop tools for performing microscopic imaging of biological and physical processes as they naturally occur in benthic ocean environments. In order to accomplish this objective, the work here combines the fields of optics, fluid mechanics, marine biology, and underwater instrument development. An outline of the structure and contributions of this thesis are presented in Table 1.1.

Chapter 2 (published in Nature Communications) describes the development and application of the Benthic Underwater Microscope. This is the first system to image benthic

marine organism *in situ* at nearly micrometer resolution. The instrument uses a custom optical design that includes a long working distance microscopic objective, an electronically tunable lens for rapid and compact focusing, and focused reflectance illumination enabling short exposures. The system is housed in a self-contained diver operated package, including an underwater computer for diver control. The instrument demonstrated the ability to resolve individual zooxanthellae inside living corals underwater. *In situ* observations with the system revealed previously un-described coordinated behavior between coral polyps. The instrument was also used to study competition between different coral species and spatial patterns of algal colonization of bleached corals.

In Chapter 3, the BUM is enhanced to perform underwater μ PTV. This is achieved by incorporating dark field illumination, which allows observations of small suspended particles in the water. The system also collects rapid image pairs using frame straddling, and particles are actively seeded into the water. Particle tracking is then applied to image pairs to retrieve two-dimensional vector fields. The instrument is validated with observations of fluid motion over a flat plate in a lab flume. *In situ* deployments demonstrate the instrument's ability to measure two-dimensional vector fields around individual coral polyps in the ocean. An approximately linear velocity profile as a function of distance from the coral surface demonstrates that the μ PTV system is observing flow within the VBL, and velocity profiles are used to estimate shear stress.

In Chapter 4, the μ PTV system is used to examine spatiotemporal variability of flow around coral polyps *in situ*. Time series data sets are used to show periodic oscillations in fluid velocities and shear stress at the coral's surface. Comparisons with nearby flow and pressure measurements indicate that these oscillations are due to small amplitude surface waves. The

expected time-scale for the DBL for growth is then compared with the wave frequency and analysis indicates that periodic peaks in velocity and shear stress are likely controlling DBL thickness and as a result mass transfer. Additionally, spatial variations in flow around individual coral polyps are investigated.

Table 1.1 | Overview of thesis structure and contributions.

Chapter 1: Benthic underwater microscope	
Motivation	• Small-scale processes can have large impacts on benthic ecosystem health. • <i>In situ</i> observations are needed to study complex ocean environments.
Completed Objective	• Underwater imaging of benthic marine organism <i>in situ</i>, at near micron resolution, performed in a minimally invasive manner.
Methods	• A custom optical system including a long working distance objective lens, an electronically tunable lens for focusing, and focused reflectance illumination enabling short exposures.
Application	• <i>In situ</i> time series observation of coral competition and coral polyp behavior. • Investigation of spatial patterns of algae colonizing recently bleached corals.
Chapter 2: Underwater micro-particle tracking velocimetry	
Motivation	• Flows at the surface-water interface control mass and momentum transfer between the benthos and the surrounding water.
Completed Objective	• Particle tracking velocimetry at micro-scales, in the ocean, at the surface-water interface of benthic organism such as corals.
Methods	• The underwater microscope is enhanced with dark field illumination, rapid image pair acquisition, active particle seeding, and particle tracking.
Application	• Two-dimensional velocity measurements around coral polyps underwater. • Direct measurements of shear stress in the viscous boundary layer over corals.
Chapter 3: Micro-scale flows over coral polyps	
Motivation	• Corals need to transfer solutes such as oxygen with the surrounding water. • Water motions at small scales control rates of mass exchange.
Questions	• Are water motions over coral polyps temporally steady or fluctuating? • How is the micro-flow environment impacted by the outer flows and waves? • Are water motions spatially homogenous, or do they vary around the polyps?
Methods	• Time series μPTV velocity measurements around coral polyps <i>in situ</i>.
Results	• Strong periodic fluctuations in shear stress at the coral's surface. • Fluctuations in near surface flows driven by wave oscillations in outer flow.

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Chapter 2

Underwater microscopy for *in situ* studies of benthic ecosystems

ARTICLE

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Underwater microscopy for *in situ* studies of benthic ecosystems

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Microscopic-scale processes significantly influence benthic marine ecosystems such as coral reefs and kelp forests. Due to the ocean's complex and dynamic nature, it is most informative to study these processes in the natural environment yet it is inherently difficult. Here we present a system capable of non-invasively imaging seafloor environments and organisms *in situ* at nearly micrometre resolution. We overcome the challenges of underwater microscopy through the use of a long working distance microscopic objective, an electrically tunable lens and focused reflectance illumination. The diver-deployed instrument permits studies of both spatial and temporal processes such as the algal colonization and overgrowth of bleaching corals, as well as coral polyp behaviour and interspecific competition. By enabling *in situ* observations at previously unattainable scales, this instrument can provide important new insights into micro-scale processes in benthic ecosystems that shape observed patterns at much larger scales.

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The health and long-term dynamics of coastal ecosystems such as kelp forests, mangroves, seagrass beds and coral reefs are significantly impacted by activities that occur on scales of a millimetre or less. Examples of these activities include: coral bleaching (expulsion of single-celled symbiotic algae)¹, larval attachment and survival², competition between organisms along thin interfaces^{3–5}, and fluxes of particles to and from the seafloor^{6,7}. These and other small-scale marine processes are of interest to scientists across diverse disciplines such as physiology, ecology, biomechanics and geology. However, in studying these systems it is important to consider that coastal oceans are complex and dynamic. The physical environment is continuously changing with high spatial and temporal variations in oxygen, pH⁸, temperature and fluid motion⁹. Organisms interact with each other in complex and non-linear ways across a wide range of scales¹⁰. In addition, many of these biological and physical processes are coupled and influence one another^{8,9}. It is therefore difficult to study these processes in the lab, as it is impossible to fully replicate the intricacies of these natural systems *in vitro*. As a result, a distinct need exists to make observations of important environmental processes *in situ*, under natural conditions.

Previous calls for technology to perform micro-scale imaging in the ocean include that of Smetacek¹¹ who asked ‘Could such an instrument (an *in situ* computerized telemicroscope) do for microbial ecology what Galileo’s telescope did for astronomy?’. Underwater optical systems have been developed to image seafloor environments at large scales^{12,13}, as well as to image free-floating zooplankton and larger phytoplankton at millimetre scales^{13,14}. In addition, holography¹⁵ has been used to acquire microscopic images of free-floating plankton. However, the development of underwater microscopes to image benthic seafloor organisms at close to micrometre resolution has been lacking^{13,14}. Imaging the seafloor environment presents significant additional challenges including active instrument positioning, precise focusing and reflectance illumination. Previous efforts to observe microscopic activities on the marine benthos have been significantly constrained in their potential applications due to both their highly intrusive nature and limited ability to collect spatial and temporal data^{16–18}. A fundamental deficiency thus exists in our ability to observe micro-scale biological and environmental processes as they naturally occur near the seafloor. This hinders our capacity to connect conceptual models and lab studies to real world environments. It also impedes efforts to identify underlying mechanisms that drive large scale ecosystem change^{10,19,20}.

To address these observational needs, we developed the Benthic Underwater Microscope (BUM). The BUM is an imaging system that provides the first *in situ*, underwater observations of benthic environments at nearly micrometre resolution. The diver-deployed, portable instrument can record dynamic natural processes and spatial patterns with minimal disturbance to benthic organisms or their surrounding physical environment. In addition, extended time-series recordings can be collected to reveal slow or periodic activities and processes, allowing for studies of animal behaviour (for example, individual coral polyps). These capabilities allow both temporal and spatial analysis of ecologically significant phenomena at scales never before seen in the natural environment. Here we detail the instrument’s novel design, which addresses the unique challenges of underwater microscopy. We then demonstrate the system’s capabilities with *in situ* images and videos of reef building corals. This includes time-series observations of coral behaviour and competition, as well as a quantitative analysis of *in situ* algal colonization patterns on bleached coral tissue during the 2015 coral bleaching event in the Main Hawaiian Islands.

Results

Instrument design. Attaining non-invasive, micro-scale images in an underwater environment presents several challenges. First, microscopic imaging requires a high numerical aperture; this results in a shallow depth of field that necessitates precise focusing. Second, non-invasive imaging requires a long working distance and considerations must be made for imaging live organisms with three-dimensional structure. Finally, to perform such work underwater, rapid focusing and exposures must be used due to the unstable environment. The BUM overcomes all of these challenges through the application of three principle optical components: a long working distance microscope objective lens, a shape-changing Electrically Tunable Lens (ETL) and focused Light Emitting Diodes (LEDs) providing reflectance illumination (Fig. 1a). These elements are integrated into a compact, diver-deployed imaging system that includes a camera, electronics and user interface. The instrument is divided into two housings: the imaging unit containing all optical components, and the control unit containing a computer and a live diver interface (Fig. 1a,b).

A long working distance microscope objective lens provides the magnification and numerical aperture required to resolve fine details, as well as the working distance necessary to image through an optical port while leaving the subject undisturbed. The BUM was equipped with either a $\times 3$ or $\times 5$ magnification objective. As measured using a resolution target in a testing tank, the $\times 5$ lens attained an underwater resolution of $2.2\ \mu\text{m}$ with a $1.62 \times 1.36\ \text{mm}$ FOV, while the $\times 3$ lens attained a resolution of $3.1\ \mu\text{m}$ with a $2.65 \times 2.22\ \text{mm}$ FOV (Supplementary Fig. 1). Each objective provides a working distance of $> 65\ \text{mm}$ from the optical port, allowing instrument set-up and imaging with minimal disturbance to the subject and its surrounding environment.

To achieve rapid focusing, a deformable ETL was incorporated into the optical system. This lens consists of a flexible polymer membrane encasing an optical fluid. An integrated actuator exerts variable pressure on the encased optical fluid to rapidly change the lens curvature and focal length with adjustment times of $< 2.5\ \text{ms}$ (refs 21,22). The ETL provides a compact means to bring a subject of interest into precise focus, which is a principle challenge in benthic underwater microscopy. The ETL can also rapidly scan the optical system’s focal plane through a volume to bring various parts of a subject in focus. Frames can then be combined using image-processing techniques to produce a single image with all parts of the subject in focus²³. This is commonly known as focus stacking and is an important capability for collecting observations of natural, undisturbed subjects, which often have substantial three-dimensional structure (Fig. 2). While the $\times 3$ and $\times 5$ objectives have narrow depths of field ($34\ \mu\text{m}$ and $16\ \mu\text{m}$, respectively) the ETL enables focusing over scanning ranges of $18.4\ \text{mm}$ and $6.9\ \text{mm}$, respectively.

A custom designed ring of six LEDs provides the high-intensity light required for short duration, reflectance illumination. Each LED is focused with a condenser lens and angled such that all light sources converge at the plane being imaged (Fig. 1, Supplementary Fig. 2). Using illumination pulses, images were captured in the ocean with exposure times of $< 1\ \text{ms}$, which is critical for eliminating motion blur. In addition, short LED pulses reduce the total length of time organisms are exposed to artificial illumination. While we did not observe any distinct behavioural changes due to the instrument’s illumination, if needed imaging can be performed at very low frequencies to further reduce artificial light effects. During most operations, wide spectrum white LEDs were used. However, fluorescence imaging was also conducted using blue LEDs combined with a long-pass optical filter in the imaging system (Fig. 3b), additionally near-infrared (NIR) LEDs were tested (Supplementary Fig. 3).

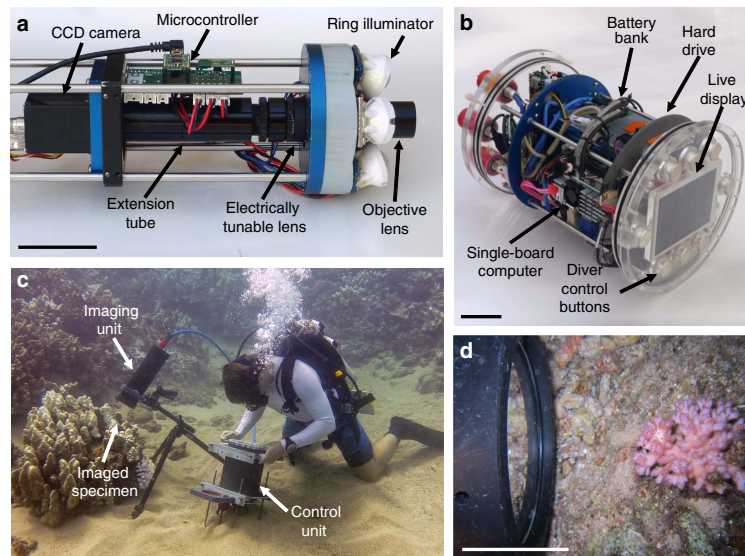


Figure 1 | BUM design and operation. (a) Imaging unit internal components including illumination ring, optical lenses, CCD camera and electronic circuit board with micro-controller. (b) Control unit internal components including user interface with live LCD display and buttons for diver control, single-board computer and battery bank. (c) *In situ* underwater operation of the BUM by a scientific diver. (d) Close up of the BUM imaging a coral colony *in situ*, the BUM maintains a distance >65 mm from the specimen during imaging. (a,b,d) Scale bars, 50 mm.

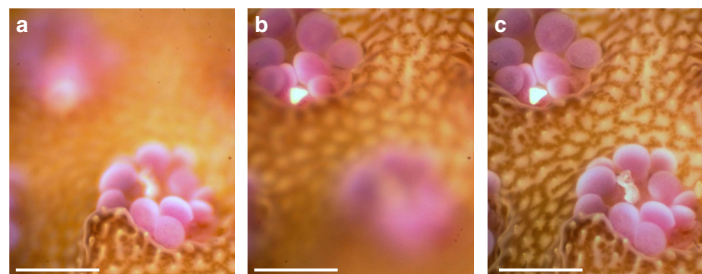


Figure 2 | Focal scan using ETL and composite image formation. Images of a live coral acquired *in situ* with the BUM using an ETL focal scan. Images collected using the $\times 5$ objective and wide spectrum white LED illumination. (a) Image of a single focal plane showing only the front coral polyp in focus. (b) Image of a single focal plane showing only the back coral polyp in focus. (c) A composite focus stacked image formed using the in-focus portions of 20 images collected with the ETL focal scan. Within a single frame (a,b), the microscope objective yields a shallow DOF. However, the composite focus stacked image, as shown in c, combines frames to provide an enhanced DOF such that both polyps and surrounding area are all in focus. Scale bars, 500 μ m.

The entire system is housed in a self-contained, submersible package, which enables underwater operation of the instrument in the ocean by a single scientific diver (Fig. 1c). The instrument's physical design consists of two cylindrical aluminium housings. The imaging unit contains all optical elements, as well as a CCD camera, micro-controller and custom circuit board. The camera's imaging rate can be adjusted in a continuous range from a maximum of 15 frames per second (for rapid focal scans) to once every several minutes (for time-series recordings and reduced artificial light effects). The control unit contains an on-board computer, 500 Gb hard drive for data storage and real-time diver-controlled user interface. The BUM has a battery capacity of ~ 8 h, enabling video and extended time-series recordings (during which the instrument can be left on the reef to image autonomously). In the videos shown here, the imaging unit was mounted on a tripod or rested directly on the seafloor.

The tripod minimizes camera movement, facilitates non-invasive observations and enables time-series recordings. Alternatively, the instrument can be used in a hand-held mode. In this case a mechanical ranging probe (Supplementary Fig. 4) may be mounted to the imaging unit to provide a physical means for estimating the correct imaging distance, the ETL can then be scanned to image a large depth.

Microscope imaging performance. The BUM operates while submerged, recording images with an optical resolution of up to 2.2 μ m. This enables observation of fine anatomical details of organisms in both the lab and field. For example, images of live coral polyps in the ocean reveal the distribution and discrimination of individual symbiotic single-celled dinoflagellates, commonly known as zooxanthellae, living inside the coral

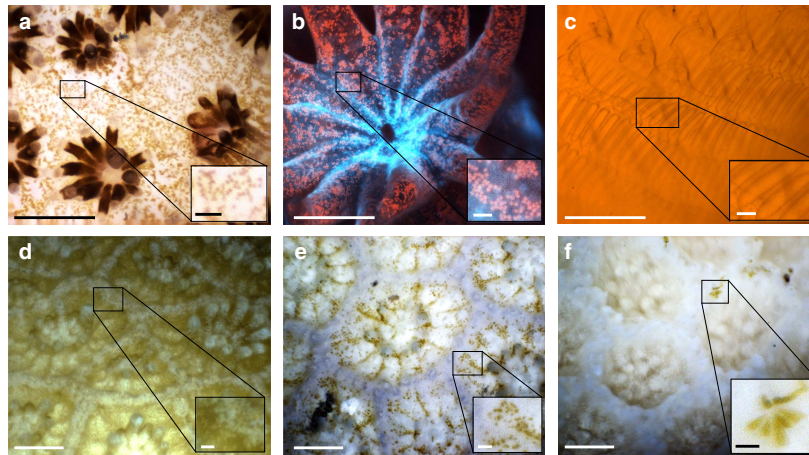


Figure 3 | Images captured by the BUM. (a) *In situ* image of the coral *Stylophora* taken using the $\times 5$ objective lens and white illumination, in Eilat, Israel. The image is an enhanced DOF composite formed from a focus stack. Individual zooxanthellae ($\sim 6\text{--}13\ \mu\text{m}$ in size) are visible in the inset. (b) Fluorescent image of the coral *Pocillopora* taken in a lab tank using the $\times 5$ objective. Inset shows individual zooxanthellae emitting red fluorescence from their chlorophyll. Image is a composite focus stack. (c) *In situ* image of the pharyngeal basket of the semi-transparent ascidian *Rhopalaea idoneta*, taken using the $\times 5$ objective and white illumination, in Eilat, Israel. *R. idoneta* is a filter feeder that uses the mesh of the pharyngeal basket to capture plankton. (The orange colour here is likely due to a subject behind the ascidian). (d,e) *In situ* images of two different locations on a bleaching colony of *Porites compressa*, taken in Maui, Hawaii. Images show partial bleaching in d and nearly complete bleaching in e. Images are composite focus stacks collected using the $\times 3$ objective lens. (f) *In situ* image of a fully bleached colony of *Porites lobata*. Taken in Maui, Hawaii with the $\times 3$ lens. No visible zooxanthellae can be seen in the coral; as a result the polyps have a translucent appearance. While translucent, the polyp structure and tentacles remain intact and visible, indicating that the polyp is still alive. Coenosarc tissue normally connecting polyps is either very thin, or may have fully retracted towards the polyps' centres exposing the coral skeleton. Inset shows colonization of the area between two live polyps by benthic diatoms. Main figure scale bars, $500\ \mu\text{m}$. Inset scale bars, $50\ \mu\text{m}$.

($\sim 6\text{--}13\ \mu\text{m}$ diameter)²⁴ (Fig. 3a). With this capacity, we examined corals experiencing varying levels of bleaching (expulsion of zooxanthellae) and checked for the presence of remaining symbionts (Fig. 3d–f). Fluorescence imaging was also conducted (Fig. 3b) to further enhance observations. Corals fluoresce due to chlorophyll in their symbionts and fluorescent proteins in the animal itself, both of which inform physiological status and health^{25,26}. Observations of other subjects, such as the plankton-trapping mesh of the ascidian *Rhopalaea idoneta* (Fig. 3c), offer opportunities to examine filter-feeding rates and biomechanics of delicate organisms. The non-invasive nature of the system allows recordings of small, fragile specimens such as the initial stages of micro algae colonizing bleached corals (Fig. 3f). Finally, *in situ* focus stacks can successfully image complex three-dimensional subjects (Figs 2 and 3a,d,e).

Time-series and video observations. Temporal observations collected by the BUM record ongoing micro-scale processes and may reveal novel phenomena. This capability is demonstrated through recordings of coral polyp behaviour, feeding and competition. During feeding studies conducted in the lab, high numbers of *Artemia* nauplii were introduced to *Stylophora* corals. Subsequent coordinated polyp behaviour was recorded in which coral polyps that had captured a high quantity of nauplii joined with neighbouring polyps by intertwining their tentacles to digest their prey (Fig. 4a–c, Supplementary Movie 1). Time-series microscopy recordings were then collected *in situ* for periods of up to 8 h to investigate the presence of these and potentially other periodic feeding activities under natural conditions. For these investigations, the BUM set-up to image autonomously on the reef overnight in the Gulf of Eilat at depths between 4 and 8 m. Here we recorded a previously undescribed behaviour, in which

polyps periodically connected their gastrovascular openings throughout the night, likely exchanging materials (Supplementary Movie 2). We referred to this activity as coral ‘polyp kissing’, and it often occurred after what may have been plankton capture events.

The utility of *in situ* time-series microscopy was further demonstrated through an observational study on coral–coral competition. Work was conducted on the reef in Eilat at depths between 4 and 8 m. Here loose coral colonies were moved in close proximity ($\sim 1\ \text{mm}$) to one another to stimulate inter-colony competition. The BUM was fitted with the $\times 3$ or $\times 5$ objective lens and images of the interaction zones were autonomously recorded over the course of the night at a frame rate of 1 Hz. Pairings of different coral genera included *Stylophora* with *Pocillopora* (Supplementary Movie 3, set-up shown in Supplementary Fig. 5) and *Platygyra* with *Stylophora* (Fig. 4d–f, Supplementary Movie 4). Videos allow elucidation of competition response times, attack mechanisms such as emission of mesenterial filaments and competitive dominance between different taxa (Fig. 4d–f, Supplementary Movies 3–5). Further manipulations and a control were also staged to examine responses to differing stimuli. The coral genus *Platygyra* was paired with four different subjects: the coral *Stylophora*, the coral *Galaxea*, a conspecific *Platygyra* colony and a mesh net filled with *Artemia* (Supplementary Movie 5, Supplementary Fig. 6). Time-series videos revealed different behaviours of individual polyps when paired with different species or with conspecifics. For example, *Platygyra* quickly emitted its mesenterial filaments when paired with the coral *Galaxea*, while alternatively no aggressive behaviour or even contact was observed when it was paired with a conspecific. Observations in the natural environment of such behaviours at these spatial and temporal scales are not possible with any other available imaging system.

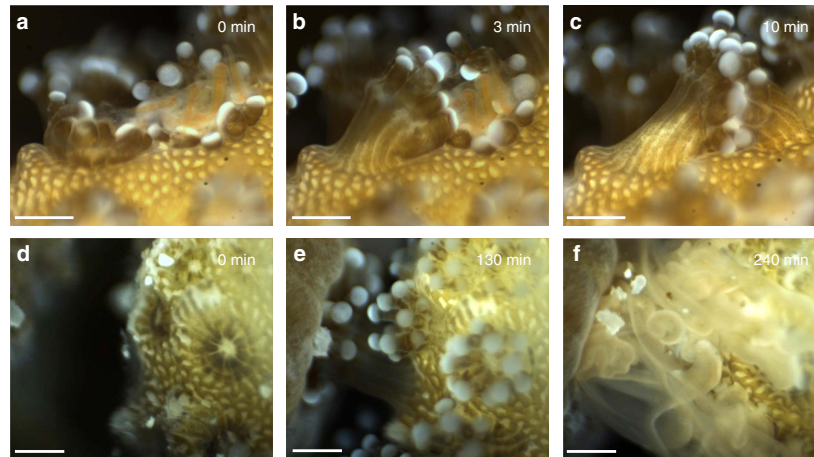


Figure 4 | Videos captured by the BUM. (a–c) Lab images of the coral *Stylophora* showing coordinated behaviour and communal feeding between two adjacent polyps after several *Artemia*, which were injected into the tank, had been captured by the polyp on the right. Image taken using the $\times 3$ objective, more details in Supplementary Movie 1. (d–f) *In situ* images of competition between the corals *Platygyra* (left side of images) and *Stylophora* (right side of images) collected using the $\times 3$ objective. The *Platygyra* has emitted its mesenterial filaments and is beginning to digest the *Stylophora* in f. More details shown in Supplementary Movie 4. Figure scale bars, 500 μm .

Spatial pattern analysis. In addition to performing temporal recordings, the instrument can also collect images for high-resolution spatial analysis. The analysis of spatial structure is a fundamental tool used to infer underlying processes in disciplines such as ecology and physiology^{10,27–32}. However, observations must be collected at scales relevant to the subject (organism or function) of interest. Via its high resolution, the BUM offers a means to collect such spatial information at new scales. Here we demonstrate this capability by examining the micro-scale spatial patterns of algae colonizing corals undergoing bleaching.

During the summer and fall of 2015, the Main Hawaiian Islands experienced their first recorded mass coral bleaching event. Bleached corals lost their symbiotic zooxanthellae, but remained alive, with polyps still visible (Fig. 3d–f). However, following bleaching, many corals on the island of Maui experienced rapid overgrowth by communities of benthic diatoms and turf algal filaments (Supplementary Fig. 7). This overgrowth was associated with the decline and ultimate death of these colonies (Supplementary Fig. 8). The algal overgrowth of bleached corals while still alive has rarely been documented at such significant magnitudes. Relatively little is known regarding the specific patterns and mechanism by which these algae are rapidly colonizing and overgrowing living corals^{33,34}. In addition, lab techniques for investigating such small-scale interactions would require destructive sampling to retrieve specimens.

To more closely examine this process in its natural, undisturbed state the BUM was used *in situ* to image communities of benthic diatoms and turf algal filaments growing on bleached *Porites lobata*. Varying successional states of algal colonization were observed by imaging different locations on the corals' surfaces (Fig. 5a–e). *In situ* images show algae colonizing the surfaces of living corals in which polyps were clearly visible (Figs 3f, 5a,c; Supplementary Fig. 9). Images were used to investigate whether algal colonization of bleached corals was occurring in a random or non-random pattern. First image-processing techniques were applied to segment algae, then algal spatial distributions were analysed using $g(r)$, the pair-correlation function (PCF) (Fig. 5) (see Methods)^{28–32}. The $g(r)$ value is a measure of the relative density of neighbouring conspecifics

(similar individuals) around an average individual as a function of radius, r . This metric allows analysis of clustering at multiple independent length scales. If more neighbours are present at a given radius than would be expected with a random arrangement then, $g(r) > 1$, indicating clustering (aggregation). Alternatively if fewer subjects are present than expected then $g(r) < 1$, indicating regularity (overdispersion) (details in Methods).

The PCF analyses show strong patterning in $g(r)$, indicating high levels of spatial order that demonstrates the algae are not randomly arranged^{29,30}. Specifically, the PCF plots indicate three critical scales of interest (Fig. 5k–o). At small scales, r less than $\sim 300 \mu\text{m}$, high $g(r)$ values denote significant aggregation. At intermediate scales, r in the range of $500\text{--}700 \mu\text{m}$, $g(r)$ values reach a distinct minimum around which there is a relatively symmetrical dip. Finally at larger scales, r greater than $\sim 700\text{--}900 \mu\text{m}$, $g(r)$ returns to values much closer to randomness ($g(r) = 1$). In summary, the PCF reveals a mixed pattern: algae are clustered with high densities at small scales, experience a zone of relative exclusion with low densities at intermediate scales and then return to moderate densities at larger scales. This structure suggests a pattern formed by interactions between the algae and coral. Strong small-scale aggregation indicates that algal communities grow in patches. The subsequent dip and distinct minimum in $g(r)$ reflects the existence of an exclusionary zone, occurring at a regular interval, in which algal densities are significantly lower than expected under randomness²⁹. The distance to this $g(r)$ minimum is slightly larger than the radius of a *Porites* polyp, and is likely indicative of algae clustering at the edge of a polyp with their presence being inhibited close to the polyp centre. Finally, the increase in $g(r)$ at larger radii suggests that algae patches occur separated by gaps, for example, on either side of a polyp. Comparing PCF plots, the general three-phase pattern is observed in Fig. 5a–d; however, the intensity of $g(r)$ patterning decreases with increasing algal density. The results of this preliminary *in situ* microscopy investigation show that algae can colonize living bleached corals and suggests that they may do so by establishing clusters on the ridges between adjacent coral polyps, resulting in the honeycomb pattern observed in Fig. 5. Further, these images demonstrate the BUM's ability to collect

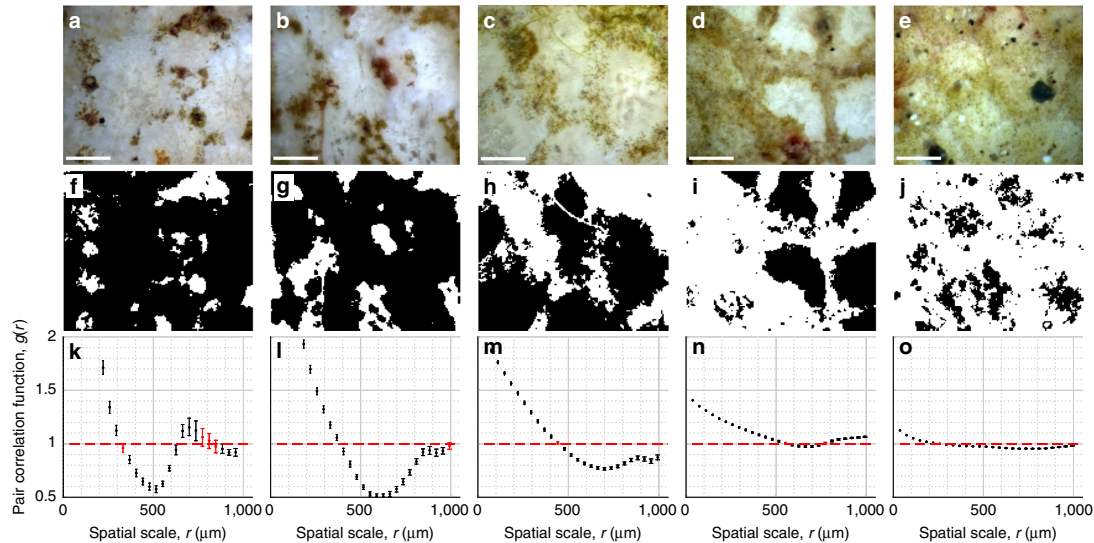


Figure 5 | Spatial analysis of algal overgrowth on bleached coral. (a–e) *In situ* images acquired at the Kahekili reef site in West Maui showing different successional stages of a filamentous algal overgrowth on bleached *Porites lobata*. All images were captured on the same dive. The $\times 3$ objective lens was used providing a 2.82×2.36 mm field-of-view. The structure of live coral polyps can be seen on close examination in a,c, polyps are dead in e and images are inconclusive for b,d. Algae are on surface (not endolithic) as they were observed swaying in the water over several images. (f–j) Categorical raster maps showing locations of algae represented by white cells. The raster map consists of 153×128 grid cells (each $18.4 \times 18.4 \mu\text{m}^2$ in size) each representing the presence or absence of algae. Algae segmentation was performed using a global saturation threshold. (k–o) Pair-correlation function, $g(r)$, values calculated using the algal raster map (details in Methods); $g(r) > 1$ indicates clustering, and $g(r) < 1$ indicates regularity, where radius r is the spatial scale being considered. The $g(r)$ values include 95% confidence intervals, which were calculated using a method of resampling without replacement³⁰. Each confidence interval was determined from the distribution of $g(r)$ values calculated from 999 resampling draws of half the population (see Methods). $g(r)$ points are red if the confidence interval overlaps with $g(r) = 1$ (which is consistent with complete spatial randomness (CSR)), otherwise points are black indicating statistically significant deviation from CSR. The strong patterns in $g(r)$ indicate spatial order. At small scales, algae are strongly clustered, there is then a distinct dip and recovery in $g(r)$, indicating a region with reduced algal density, likely due to exclusion by coral polyps. Figure scale bars, $500 \mu\text{m}$.

ecologically significant spatial data that can be used for quantitative analysis.

Discussion

Using the novel BUM, we have demonstrated the first *in situ* imaging of benthic marine organisms at nearly micrometre resolution. The methods described here provide the means for rapid exposures, active focusing and a long working distance. This facilitates non-invasive microscopy of live three-dimensional subjects in the ocean. Several scientific applications of the system were demonstrated through field observations on coral reefs. The instrument enabled time-series recordings of new organism behaviour, as well as observations of important biological patterns at heretofore unobserved scales.

In situ microscopy videos revealed novel coral behaviours, where individual polyps periodically connected their gastrovascular openings in what is likely to be a mechanism for resource sharing. While previous studies have demonstrated transfer of energetic products via connective tissue between polyps³⁵ additional resource sharing mechanisms have been hypothesized³⁶ but not directly observed. In addition, we document detailed interactions within and between coral species, including the competitive dominance of certain taxa. These interactions could be further studied with more replicated observations. The recordings show that corals have the capacity to differentiate between conspecifics and other competitors, highlighting several interesting hypotheses (such as chemical,

microbial interactions) that could be tested using the BUM with controlled *in situ* experiments^{37,38}.

The system's ability to collect meaningful spatial data was demonstrated through *in situ* microscopy of bleached corals that were being colonized by filamentous turf algae. To characterize the algae's micro-scale spatial patterns, the images were analysed using a second order spatial statistics approach (PCF). The analysis supports the hypothesis that algae are distributed in significant patterns, and that these patterns can be statistically observed within single microscope images. Further, the initial stages of succession appear to be characterized by algae clustering in patches between coral polyps. Although more complete studies with repetition would be necessary to completely validate this interpretation, these initial observations merit some mechanistic consideration. First, the exclusion of algae from large gaps indicates that, as we interpret it, little or no algae may settle directly on live polyps³⁴. The clustering between polyps also suggests that the thin coenosarc tissue connecting polyps may be weakened or retract during bleaching (such as observed before polyp bailout³⁹), exposing the underlying skeleton and providing algae a critical surface on which to initially settle⁴⁰. However, it is important to note that alternate processes may also generate the observed patterns so further testing is needed to confirm any specific mechanisms. Finally, algae distributions may exhibit systematic changes with increasing density; such as increasing algal patch connectivity, resulting in the isolation of individual polyps. Further sampling and analysis offer a means to reach quantitative conclusions regarding changes in algal spatial

distributions as function of density. Here we examined one important case of coral–algal interaction, in alternate cases patterns of algal colonization and succession may vary considerably based on the species of coral and algae involved, as well as the conditions they are exposed to ref. 33. However, most importantly for the discussion here, results demonstrate that the BUM can record images that can be processed to obtain quantitative *in situ* spatial data for testing specific hypotheses.

Natural processes occurring in benthic marine environments span extensive spatial and temporal scales (Supplementary Fig. 10), and in many cases small-scale processes drive the structure and health of the larger ecosystem¹⁰. For example, while corals create massive reefs that can be viewed from satellites, individual coral polyps are typically on the order of a millimetre in size. In terrestrial ecology, the principle of the ‘plant’s eye view’ of a community has been used to emphasize the importance of studying subjects at scales relevant to individual organisms and the local environments they directly experience^{32,41}; here we enable that perspective for marine subjects, including a ‘coral polyp’s eye view’. Previous observations of micro-scale benthic marine processes have been confined to the lab, imposing significant limitations on what can be observed. Underwater microscopy thus offers a new means to study behaviours and interactions in the natural environment that may otherwise remain unresolved or poorly understood. This is of heightened importance as coral reefs around the world are facing increasing stress from anthropogenic activities, resulting in declines in coral cover on many reefs^{3,19,20}. However in many cases, the mechanisms and details of these declines are not fully understood. The BUM has potential to provide new insights on diverse ecological topics directly relevant to these declines such as competitive dynamics at the thin algal–coral interfaces^{3–5}, coral bleaching and recovery^{1,42}, disease dynamics^{43,44}, and larval settlement and growth. As a versatile tool, underwater microscopy has clear and viable applications in a wide variety of scientific fields including ecology, physiology, biomechanics^{2,45}, fluid dynamics, marine geology and physical-biological coupling^{7,46}.

Finally, the instrument offers a platform for technology development. A variety of enhanced imaging methods may be incorporated into future designs to provide additional information on micro-scale physical and biological phenomena. Such techniques include variable chlorophyll fluorescence imaging to study photosynthetic efficiency²⁵; micro-particle image velocimetry to study small-scale fluid dynamics^{47,48}; chemical measurements using optical indicators^{25,49}; and three-dimensional modelling of imaged subjects using the depth information that is available from the ETL focus stacks. We hope that insights from the current instrument design and deployment methods will provide a foundation for both increasingly powerful *in situ* imaging, and a wave of bringing lab research into the ocean. This *in situ* viewpoint enables novel investigations on basic marine research and will provide the means to connect theoretical lab work to the natural environment.

Methods

Optical system. The optical system is modular and accommodates standard C-mount long working distance objective lenses. Here we use two different telecentric finite conjugate objectives (Mitutoyo ML $\times 5$, ML $\times 3$). Light entering the microscope passes from the water through an acrylic optical port (5.6-mm-thick) and into the objective lens. The numerical aperture ($NA = n \times \sin(\theta)$), where n is the index of refraction and θ is the half angle of the light cone entering the lens) of the optical system is conserved across the port’s flat-refracting boundaries, following Snell’s Law. However, the instrument’s working distance increases due to water’s higher index of refraction.

The shape-changing ETL (Optotune EL-10-30-C) provides fast electrically controlled changes in focal length. The ETL has a back focal length range of 20–200 mm. It can be paired with a ~ 150 mm meniscus offset lens such that the

combined lenses have a focal length range of ~ 130 –50 mm. The optimal position for the ETL is the back focal plane of the microscope objective. However this plane was inaccessible, so the ETL was positioned just after the objective lens. As a result, the size of the FOV changes by $\sim 13\%$ in both width and height over the focal scan. The FOV can be adjusted in post processing for focus stacks so that all images are the same size.

Images are recorded by a machine vision Prosilica GC2450 colour CCD camera (12 bit dynamic range, $2,448 \times 2,050$ pixels, $3.45 \mu\text{m}$ pixel pitch, maximum frame rate of 15 fps at full resolution, global shutter). The ring illuminator (Supplementary Fig. 2) contains six LEDs with emission wavelengths for either white light reflectance illumination (LED Engin LZ1-00WW00, warm-white), NIR reflectance illumination (LED Engin LZ1, far-red peak at 740 nm) or fluorescence (LED Engin LZ1-00DB00, peak at 460 nm) imaging. Each LED is focused using an aspheric condenser lens (27 mm Diameter $\times$ 13 mm FL, Edmund Optics 43–987). The illumination ring and condenser lens mounts were fabricated using three-dimensional printing. During fluorescence imaging band pass excitation filters, transmitting between 419 and 465 nm (SemRock, CFW-BP01), are mounted on the LEDs and a long-pass emission filter, cutoff of 473 nm (SemRock, BLP01-473R), is mounted behind the objective lens.

Housings and electronics. The housings for the control unit (30.6 cm length $\times$ 21.9 cm diameter) and imaging unit (39.5 cm length $\times$ 12.2 cm diameter) were constructed from 6061 aluminium cylinders. End caps were machined from optically clear sheets of Spartech Polycast Super Abrasion Resistant acrylic (5.6-mm-thick for the optical housing and 25-mm-thick for the control housing). The housings were pressure tested to a depth of 30 m, sufficient for normal SCUBA operations. In addition pressure ratings of up to 300 m can be achieved by using thicker end caps. A diving frame machined from PVC sheets holds both housings during underwater deployments. The entire system weighs ~ 23 kg in air, but is only slightly negatively buoyant underwater.

The control unit houses a Pico-ITX embedded motherboard (LP-170, Global American), 500 Gb solid-state hard drive and four Li-Ion batteries (Ocean Server) permitting ~ 8 h of system operation. Real-time, underwater user control is achieved through a 5 inch LCD screen (Purdy Electronics), and eight piezo-electric buttons (Baran Advanced Technologies). A graphical user interface enables live viewing of acquired images and full instrument operational control; software was written in C++ and based on the camera’s SDK. The control and imaging units are connected via a 2 m underwater cable (SubConn) supporting Ethernet, USB and power connections.

The opto-electronics in the imaging unit are controlled by a micro-controller (Teensy 2.0, Arduino code) mounted on a custom PCB. This system synchronizes the camera shutter, illumination LEDs and the ETL. The ETL’s focal length changes as a function of a supplied current, which is adjusted by a precision current controller (ADN8810) mounted on the PCB. The PCB also supports two LED illumination modes: a high current supplied during imaging for pulsed illumination, and a low current supplied for low intensity continuous illumination. The control unit’s motherboard is connected to the imaging unit’s micro-controller via USB and the CCD camera via Ethernet.

Image processing. Image focus stacks were combined to form enhanced DOF images using a commercial software package (Helicon Focus). For display, linear image contrast stretching and brightening was applied. In addition, specular reflections in resolution target images were eliminated by applying a threshold to reduce anomalously bright spots.

The videos were processed using an open source software package (Virtual Dub). This package was used to stabilize Supplementary Movie 3; additionally small smudges on the camera sensor were removed from Supplementary Movies 1, 3 and 4. Finally, linear image contrast stretching and brightening were applied. Videos were compressed using the open source FFmpeg software package.

Lab measurement of optical performance. The instrument’s resolving power was quantified in the lab using a 1951 USAF resolution target mounted on a white PVC sheet. The resolution target was designed for transmission illumination (we were unable to find a target designed for reflectance imaging with small enough resolution bars), and as a result target images may show reduced contrast compared with other specimen. Imaging performance tests were conducted in a test tank filled with seawater, as well as in air. The resolution target was positioned using a micromanipulator with a movement accuracy of 0.025 mm. Maximum resolution in seawater was measured to be $2.19 \mu\text{m}$ with the $\times 5$ lens and $3.11 \mu\text{m}$ with the $\times 3$ lens (Supplementary Fig. 1).

It was also necessary to consider two sources of changing aberrations in the system caused by the ETL. First, the flexible membrane of the ETL lens sags slightly due to gravity, creating asymmetry in the lens. Thus for optimal imaging, the ETL should be placed in a vertical position, with the optical axis parallel to the gravity. In the horizontal position the BUM’s underwater resolution decreased to $2.46 \mu\text{m}$ with the $\times 5$ lens and $3.91 \mu\text{m}$ with the $\times 3$ lens. Second, objective lenses are designed for operation at a specific working distance. However, as the ETL focal distance changes it moves the objective lens away from its designed working distance. This results in reduced image resolution and contrast. Resolution at the largest ETL deformation was measured to be $3.47 \mu\text{m}$ and $4.39 \mu\text{m}$ for the $\times 5$ and

× 3 lenses, respectively, largely due to decreased image contrast. Finally, the system's FOV was measured using a Ronchi ruling with five lines per millimetre, and working distance and scan range were measured using the micromanipulator to control the target's location. Values are reported in Supplementary Table 1.

Underwater operation. The BUM is designed for deployment and operation in the ocean by a scientific diver. To facilitate optimal functionality the instrument is self-contained, slightly negatively buoyant underwater, and split into two modules. The imaging unit is smaller to enable easy maneuvering and positioning. It is tethered to a larger control housing, which is separate to avoid inadvertent movement of the system's optics during user control. Challenges during the diver operation include positioning the imaging unit at the correct working distance and adjusting the desired field-of-view. With this in mind, the instrument provides an LCD display with live feedback. In addition, the six LEDs in the ring illuminator can provide continuous low intensity illumination. All six light sources are focused and converge at the point being imaged (Supplementary Fig. 2). This provides a visual aid for ranging and framing the subject of interest. Finally, fine focusing is conducted by controlling the ETL.

During time-lapse recordings and continuous imaging the imaging unit was mounted on a portable tripod (Vanguard Alta Pro 263AGH), which stabilized the instrument and provided several degrees of freedom (including a critical ball mount pistol grip). The tripod was not designed for underwater applications and eventually failed after ~25 dives due to saltwater exposure, future efforts will likely include the fabrication of a similar tripod using saltwater-resistant materials. Using this set-up, the organism is never physically disturbed and divers were able to complete tripod and instrument positioning within ~5 min, after which many images of a site can be acquired. In addition, the system was left to perform overnight imaging on the reef autonomously. Alternatively, the imaging unit can be used in a hand-held mode. In this case, a mechanical ranging probe (Supplementary Fig. 3) can be mounted around the imaging port. The ranging probe comes in contact with the substrate around the subject being imaged to indicate approximate working distance and aid instrument stability. A complete focus stack is then rapidly acquired using the ETL and the in-focus planes are selected later in the lab. This method is invasive and not ideal for fragile organisms such as corals, but can be useful for other subjects growing on firm substrates such as rocky reefs.

Spatial pattern analysis. The spatial patterns of algae colonizing bleached corals were analysed using the PCF. The PCF, $g(r)$, is a noncumulative neighbourhood density function, where r is the distance between two neighbours. It is determined by counting the number of neighbours surrounding each subject in annuli of constant width and increasing radius. Densities within each annulus are determined by dividing neighbour counts by the annulus area. Annulus densities for a given radius, r , are then averaged for all subjects. Finally, averaged densities at each radius are normalized by the average density of the entire image to obtain $g(r)$ values^{28,30,32}. This metric represents relative density as a function of distance. It is well-suited to reveal mixed patterns of aggregation and dispersion at different critical scales. It is relevant to note that while the PCF is used here, a host of spatial statistics for a variety of applications exist and may be applied to future microscopy images²⁷.

To apply the PCF, we segmented algae from the rest of the microscopic image (Fig. 5f–j). First, 2×2 neighbourhoods of pixels were averaged to reduce noise in raw images. Then, the colour space was converted from RGB to HSV. Finally, a global saturation threshold was applied to distinguish pixels occupied by algae. Saturation indicates the degree to which a given pixel's colour channels vary in intensity. Bleached coral tissue reflects light approximately evenly, producing low pixel saturation values. Alternatively, algae exhibit preferential light absorption at specific wavelengths, producing higher pixel saturation values. An expert user manually selected a saturation threshold value of 0.25 and this value was applied consistently across all segmented images. (To demonstrate that final results were not highly sensitive to small changes in the threshold value selected, we also applied the PCF analysis to images segmented using saturation thresholds of 0.22 and 0.28. The $g(r)$ values produced when using these thresholds differed by an average of <4%, from the $g(r)$ values produced when using the 0.25 threshold). Image segmentation results were used to create a categorical raster grid with each grid cell indicating presence or absence of algae^{28,32}. A cell size of $18.4 \times 18.4 \mu\text{m}^2$ (equivalent to 16×16 camera pixels) was selected as this is the approximate size of the small algal filaments in the image (and thus the minimum resolution needed to capture features of interest). Each raster map consisted 153×128 cells. It is important to note that while a simple segmentation approach is used here, many different computational techniques may be applied in the future to examine and extract different features from microscopy images⁵⁰.

The PCF was then applied to this raster map³² using the grid-based approach presented by Wiegand and Moloney²⁸, with an annulus width equal to two cells. To prevent edge effects, a buffer zone technique was applied, in which annuli were only used if they were fully contained within the imaged area²⁸. Each $g(r)$ data point was attained using annulus counts around a minimum of at least 100 individual cells (this resulted in a total of at least 8,000 individual point-to-point distances being used to calculate each $g(r)$ value). Second order spatial statistics (such as the PCF) typically operate under assumptions that the field being studied

is isotropic and homogeneous (intensity of pattern does not vary over image). However, because the PCF is a scale-dependent density function, it is less sensitive to effects of non-homogeneity than cumulative second order statistic such as Ripley's K -function; and the pattern of $g(r)$ itself can be used to detect non-homogeneity²⁸. A $g(r)$ value that trends to 1 for large scales indicates homogeneity²⁹; this is observed in Fig. 5a,b, which also show the most distinct spatial patterns. However, in Fig. 5c,d values of $g(r)$ deviate slightly from $g(r) = 1$ at large scales, this indicates that these plots may be somewhat biased by non-homogeneity, as a result their patterns may be influenced by virtual aggregation²⁸.

To estimate confidence intervals, a resampling approach was applied based on the method and interpretations presented by Condit *et al.*³⁰, as follows here. For each radius, r , a random sample of half the population was repeatedly drawn without replacement and its $g(r)$ calculated. This was repeated for a total of 999 resampling draws of half the population. A 95% confidence interval was then established by selecting the 25th lowest and 25th highest $g(r)$ values of this sampling distribution. Clustering or regularity was inferred if confidence intervals were either entirely above or below $g(r) = 1$, which is consistent with complete spatial randomness (a homogeneous Poisson process). Distance classes were judged as different if confidence intervals did not overlap³⁰. All image processing and statistical analyses were performed using Matlab.

Data availability. All data supporting the biological findings of this study are available within the article and its Supplementary Information Files. Original and full resolution images are available from the corresponding author on request. Data supporting the optical resolution of the system are contained within the Supplementary Images; additional calibration images are available from the corresponding author upon request.

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Author contributions

J.S.J. conceived the project. A.D.M. and T.T. jointly built the system, performed lab tests and initial *in situ* tests. T.T. led the design of the system with assistance from A.D.M. A.D.M. led system calibrations, field experiments and data analysis. P.L.D.R. helped and advised throughout the system design and construction process. E.L.A.K., J.E.S. and R.H. contributed to experiment design and field data collection. A.D.M., T.T. and J.S.J. wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

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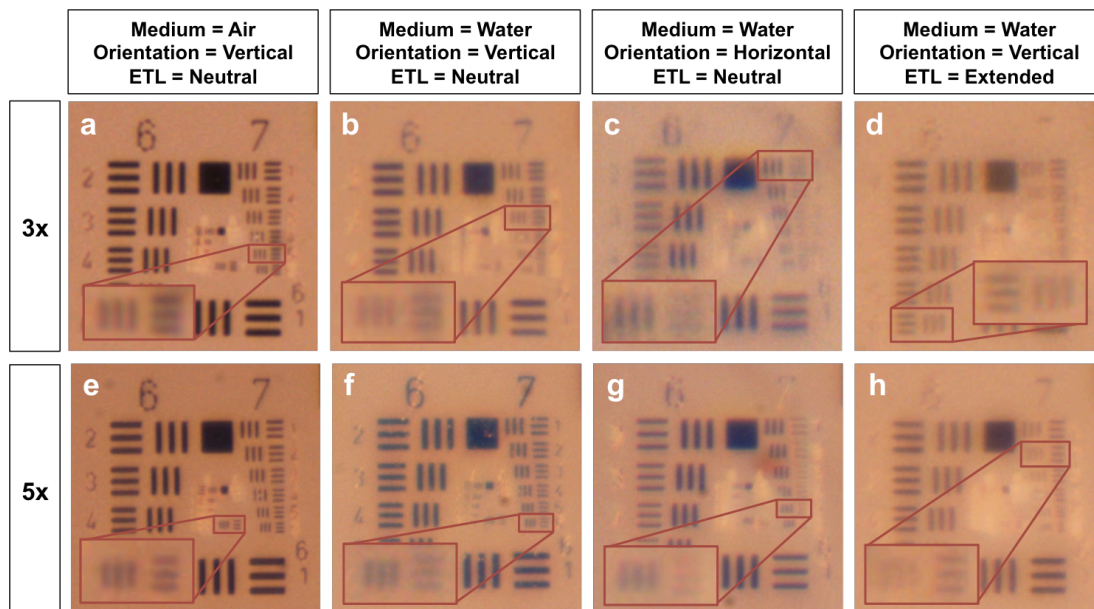
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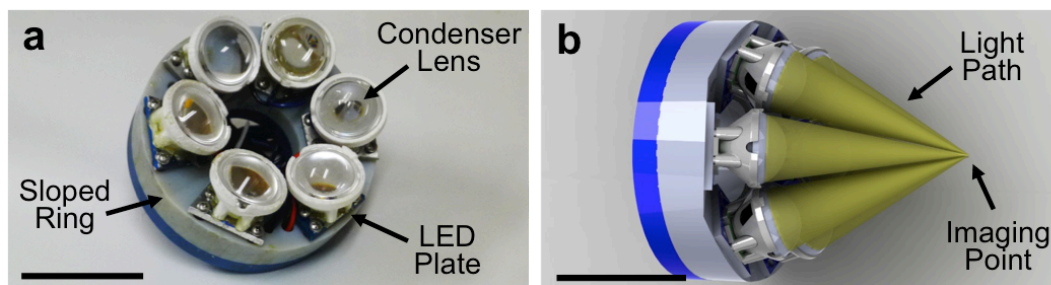
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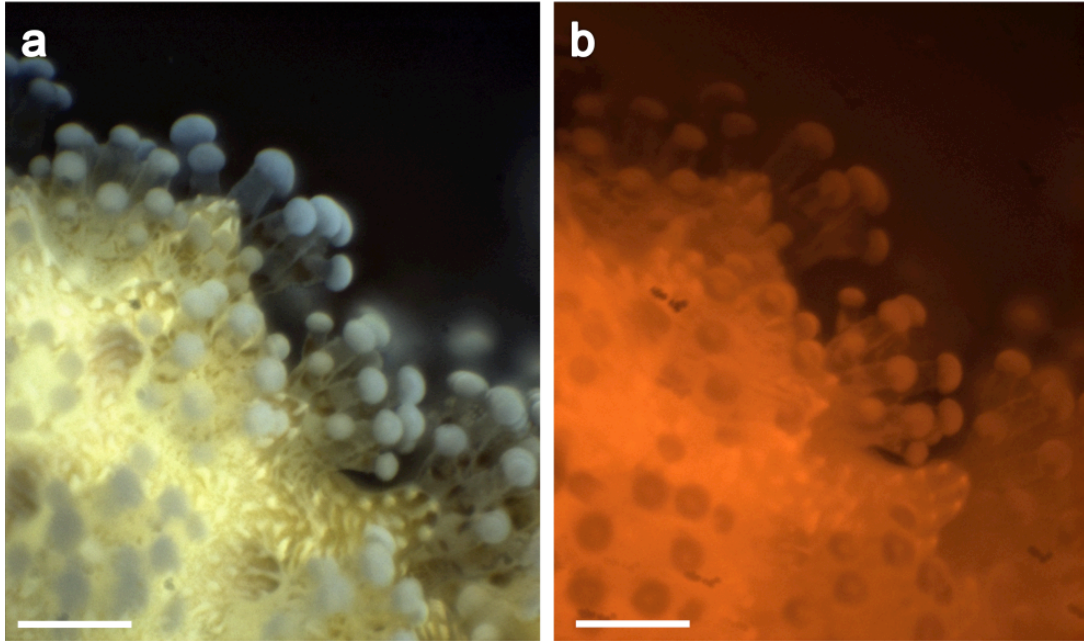
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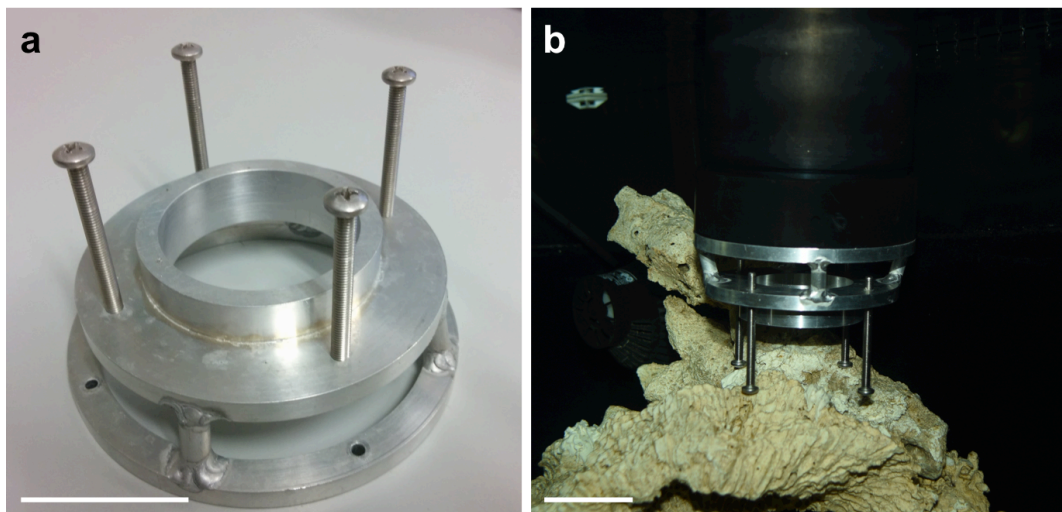
Supplementary Figure 1 | Resolution target. Images of a 1951 USAF calibration target taken with the BUM under different imaging conditions using the 3x and 5x objective lenses. Insets zoom in on the smallest resolvable bars of each test image. (a,e) Images in air with the BUM imaging unit placed in a vertical position and the ETL in a neutral setting. (b,f) Images in water with the BUM imaging unit placed in a vertical position and the ETL in a neutral setting. (c,g) Images in water with the BUM imaging unit placed in a horizontal position and the ETL in a neutral setting. (d,h) Images in water with the BUM imaging unit placed in a vertical position and the ETL in an extended setting. The highest underwater resolution for the objective lenses was attained in **b** and **f**, where the instrument was oriented vertically and the ETL set to a neutral state. Under these conditions the 3x lens was able to resolve group 7 element 3 (3.10 μm bar width) and the 5x lens was able to resolve group 7 element 6 (2.19 μm bar width).



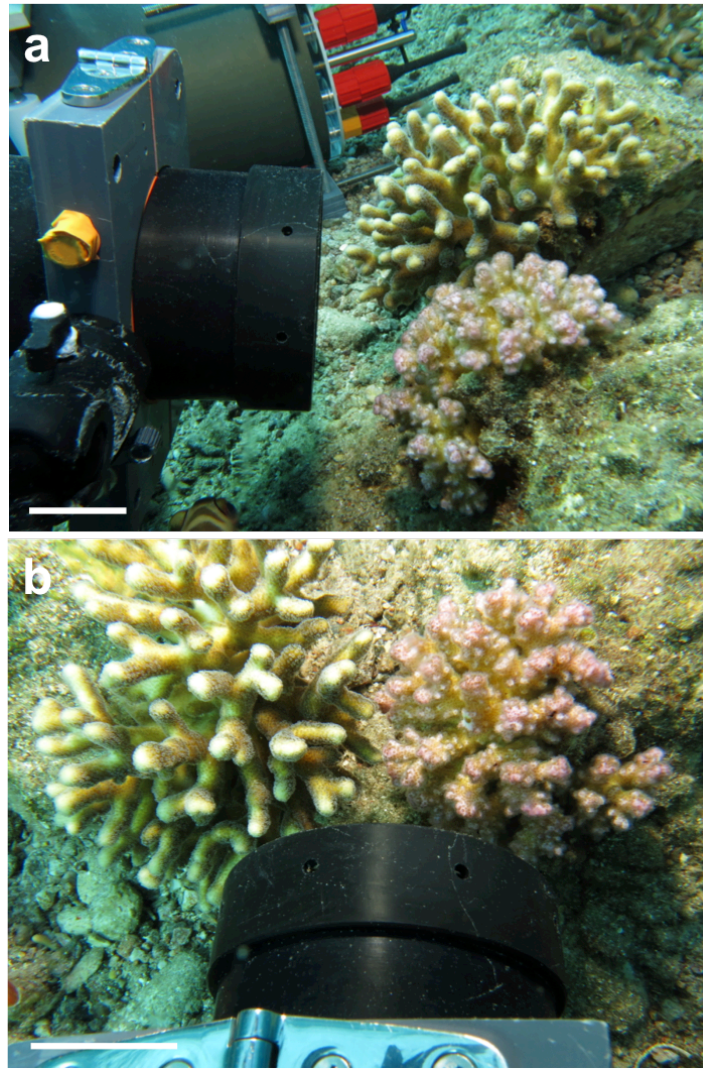
Supplementary Figure 2 | BUM illumination ring. (a) Image of the illumination ring, the design consists of 6 angled LEDs each focused with a condenser lens. (b) Digital rendering of the illumination ring, showing light paths of all LED's converge at the imaging plane.



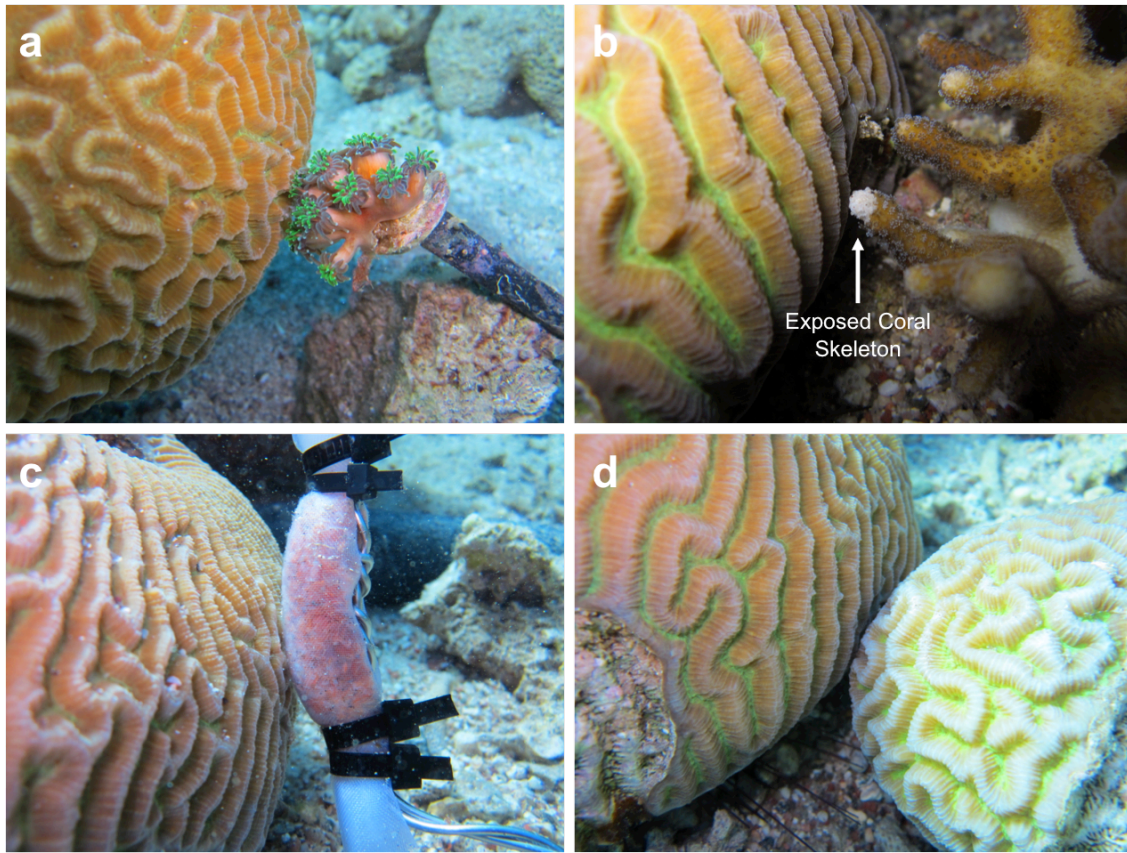
Supplementary Figure 3 | Near-IR illumination . *In situ* image of the coral *Stylophora* taken using the 3x objective lens. **(a)** Image taken using white reflectance illumination. **(b)** Image taken using near-IR reflectance illumination. Scale bars, 500 μm .



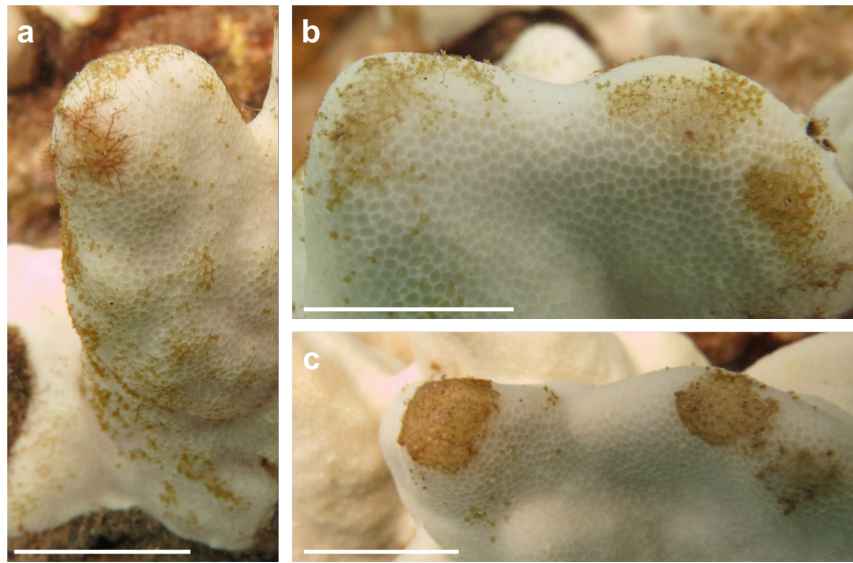
Supplementary Figure 4 | Ranging probe. A mechanical ranging probe may be mounted around the imaging port in order to facilitate handheld instrument operation. The probe can be pressed against the substrate being imaged, such as a rocky reef, in order to provide the optics housing stability and correct ranging. **(a)** Ranging probe. **(b)** Ranging probe mounted to the imaging unit, and its use being demonstrated in an aquarium. Scale bars, 50 mm.



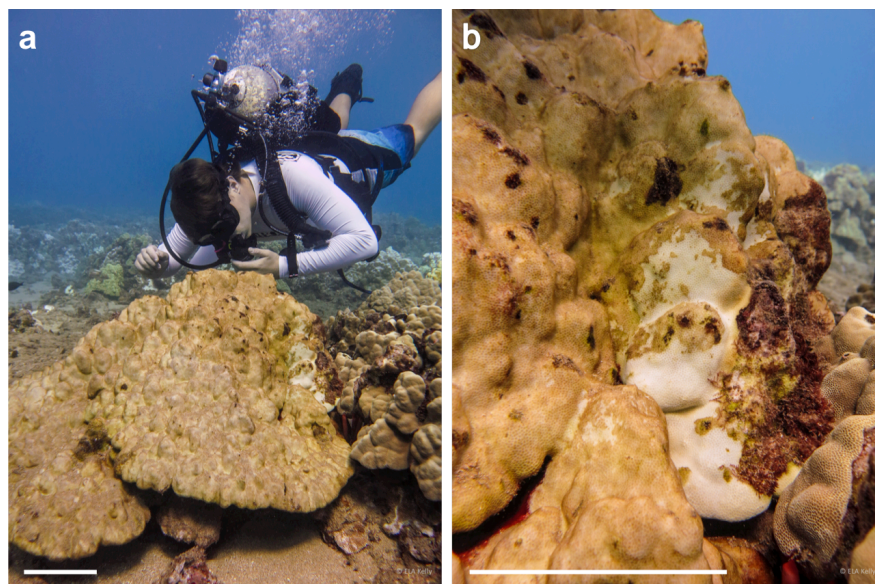
Supplementary Figure 5 | Coral competition video recording setup. (a-b) *In situ* setup of the BUM during the recording of Supplementary Movie 3. Loose coral colonies of *Stylophora* (left coral in **b**) and *Pocillopora* (right coral in **b**) were moved in close proximity (approximately 1mm) to one another. The BUM maintains a distance of greater than 65mm from the interaction area imaged. Once set in place, the BUM was left to image the coral's interaction autonomously over the course of a night. Scale bars, 50 mm.



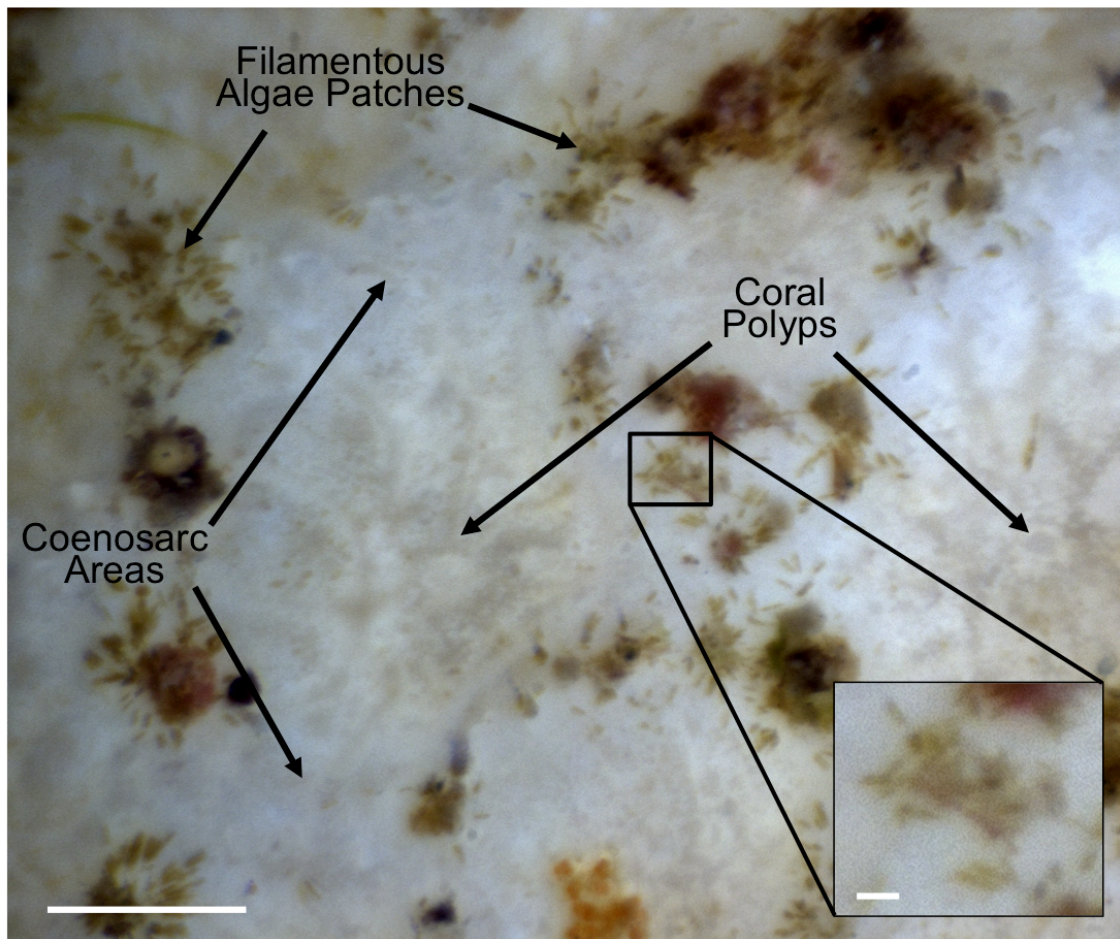
Supplementary Figure 6 | Coral *Platygyra* paired with four different stimuli. Macro images of the *in situ* organism pairings shown in Supplementary Movie 5. All images were taken after the interactions recorded in Supplementary Movie 5 had occurred. **(a)** The coral *Platygyra* paired with a small colony of the coral *Galaxea* that was brought from the lab. During the previous night the corals competed (Supplementary Movie 5). No damage to either coral is clearly visible in this image, however upon removal one of the *Galaxea* polyps was observed to have been killed. **(b)** The coral *Platygyra* paired with a loose colony of the coral *Stylophora*. The *Stylophora* colony was moved from a nearby site on the reef. The tissue on the tip of one of the *Stylophora*'s branches was digested by the *Platygyra*'s mesenterial filaments during the night (Supplementary Movie 5). The white skeleton of the *Stylophora* is now exposed where its tissue was digested by the *Platygyra*. **(c)** The coral *Platygyra* paired with a mesh net filled with *Artemia* (brine shrimp) prepared in the lab. During the previous night the *Platygyra* used its mesenterial filaments to digest some of the *Artemia* in the net (Supplementary Movie 5). **(d)** The coral *Platygyra* paired with a loose colony of the same genus that was moved from nearby on the reef. The two colonies of the same genus displayed no aggressive behavior or even contact during the recorded microscopy video from the previous night (Supplementary Movie 5). Based on the coral's features, images **a**, **b**, **c**, and **d** have fields of view with widths of approximately 75 mm to 150 mm.



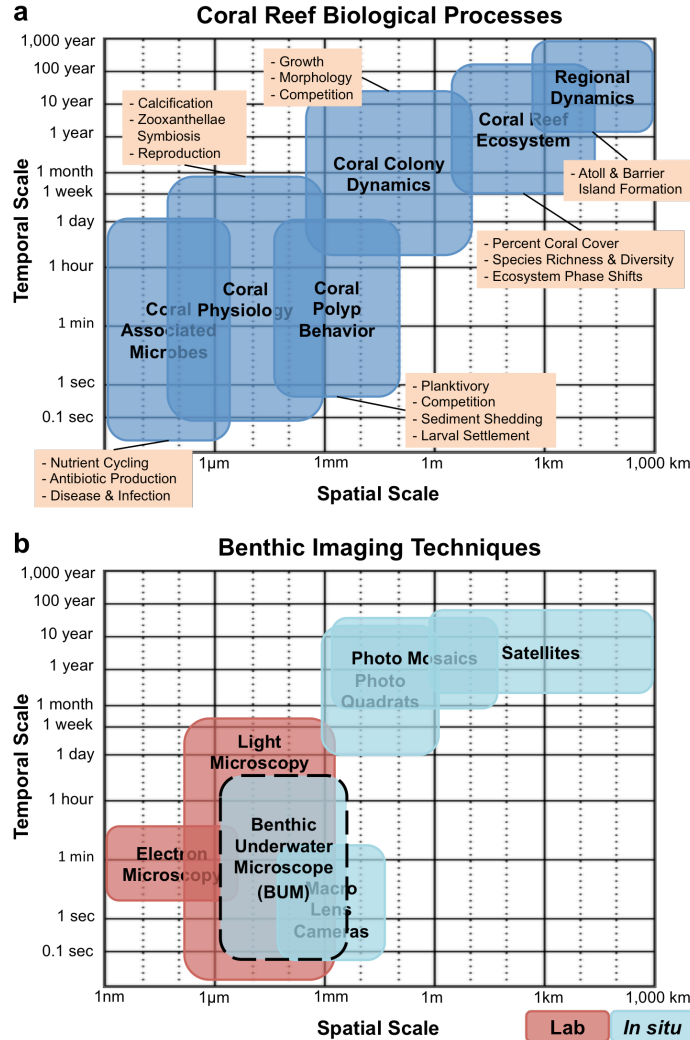
Supplementary Figure 7 | Initial algal colonization of bleached *Porites* coral colonies. (a-c) Images taken with a conventional underwater camera in Maui, Hawaii. At this stage the coral is bleached but the majority of polyps are most likely still alive. Algae exist primarily on the walls between individual coral polyps, but some small patches appear to have fully overgrown the coral's surface. Scale bars approximately 25 mm (based on size of polyps in image).



Supplementary Figure 8 | Complete overgrowth of bleached *Porites* coral by filamentous algae community. (a-b) Images taken with conventional underwater camera in Maui, Hawaii. At this stage the bleached coral colony has been almost completely overgrown by algae, and coral polyps fully covered by algae are dead. Scale bars, 250 mm.



Supplementary Figure 9 | Annotated microscopy image of algal overgrowth on bleached *Porites* coral. *In situ* image acquired with the BUM at the Kahekili reef site in West Maui using the 3x objective lens. Image shows filamentous algal overgrowth on bleached *Porites lobata*. Coral polyps are bleached (and thus translucent) but still alive. If the image is closely examined the outlines of the polyp tentacles and mouth structure are visible. Filamentous algae are forming patches around the coral polyps. The area normally covered by coenosarc tissue is indicated in the image, but it is inconclusive whether or not coenosarc tissue is still intact. Main figure scale bar, 500 μm . Inset scale bar, 50 μm .



Supplementary Figure 10 | Scales of coral reef processes. Two-dimensional Stommel diagrams showing the approximate temporal and spatial scales of coral reefs processes as well as available imaging techniques. **(a)** Approximate scales associated with important coral reef biological processes. **(b)** Approximate observational scales provided by imaging techniques used in the lab and *in situ* to investigate benthic marine organisms. Note that lab microscopy techniques typically require the isolation of small coral fragments or tissues samples. Electron microscopy techniques further require specially prepared samples that are normally fixed and no longer alive, as a result the ‘temporal scale’ of electron microscopy is not directly comparable to other techniques. Finally, while these plots show general values, the scales examined in specific individual studies may vary from those displayed here.

Supplementary Video 2.1 | Coral polyp communal feeding. Lab time series video of the coral *Stylophora* showing communal feeding between two adjacent polyps after injection of *Artemia* near the coral. Images were captured using the 3x objective (2.65 x 2.22 mm FOV) and a frame rate of 5 FPS. The video is played back at 48x live speed; the upper right corner shows the video's total elapsed time.

Supplementary Video 2.2 | Coral 'polyp kissing'. In situ time series video of the coral *Stylophora*. The video shows periodic interactions between adjacent coral polyps involving the touching of their gastrovascular cavity openings. Images were captured over a period of six hours during the night using the 3x objective (2.65 x 2.22 mm FOV) and a frame rate of 1 FPS. The video is played back at 24x live speed; the upper right corner shows the total elapsed time since video recording began.

Supplementary Video 2.3 | Coral completion between *Stylophora* and *Pocillopora*. In situ time series video of competition between the corals *Stylophora* (left) and *Pocillopora* (right). Both colonies were attached to loose rock on the reef and were moved in close proximity to each other in order to induce competition (setup shown in Supplementary Fig. 5). Images were captured at night using the 5x objective (1.62 x 1.36 mm FOV) and a frame rate of 1 FPS. The video is played back at 120x live speed, covering approximately 5.5 hours.

Supplementary Video 2.4 | Coral completion between *Platygyra* and *Stylophora*. In situ time series video of competition between the corals *Platygyra* (left) and *Stylophora* (right). The *Stylophora* colony was a loose fragment found on the reef that was moved in close proximity to the *Platygyra* in order to induce competition. Images were captured at night using the 3x objective (2.65 x 2.22 mm FOV) and a frame rate of 1 FPS. The video is played back at 480x live speed, the upper right corner shows the video's total elapsed time.

Supplementary Video 2.5 | *Platygyra* exposed to four different stimuli. In situ time series video of the interactions between colonies of the coral *Platygyra* and four different stimuli. In each frame the *Platygyra* colony is on the left side. *Platygyra* is paired with the following stimuli: top-left - a small colony of the coral *Galaxea* brought from the lab, bottom left - a mesh net filled with *Artemia* (brine shrimp), top right - a loose colony of the coral *Stylophora* moved from nearby on the reef, and bottom right - a loose foreign colony of *Platygyra* moved from nearby on the reef. Images were captured at night using the 3x objective (2.65 x 2.22 mm FOV) and a frame rate of 1 FPS. The video is played back at 480x live speed; the upper right corner in each frame shows the video's total elapsed time. Macro images of each pairing are shown in Supplementary Fig. 6.

Supplementary Table:

	Manufacturer Specifications		Seawater Tank Measurements with BUM			
Lens	Numerical Aperture	Working Distance in Air	Optimal Resolution	Field of View	Working Distance	Scan Range
Mitutoyo – 3x	0.09	77.0 mm	3.11 μm	2.65 mm x 2.22 mm	77.2 mm – 95.6 mm	18.4 mm
Mitutoyo – 5x	0.13	61.0 mm	2.19 μm	1.62 mm x 1.36 mm	67.8 mm – 74.7 mm	6.9 mm

Supplementary Table 1 | Instrument optical performance. Instrument optical performance as measured in the lab.

Chapter 2, in full, is a reprint of the material as it appears in Nature Communications 2017: Mullen, Andrew D; Treibitz, Tali; Roberts, Paul L.D.; Kelly, Emily L.A.; Horwitz, Rael; Smith, Jennifer E.; Jaffe, Jules S., Nature Publishing Group, 2016. The dissertation author was a joint primary investigator and joint primary author of this paper.

Chapter 3

Underwater micro-particle tracking velocimetry for *in situ* observation of microscopic flows over benthic organisms

3.1 Abstract

Water motions fundamentally impact the health of important benthic marine organisms such as corals and kelps. Micro-scale fluid dynamics at the surfaces of such organisms are especially important as these regulate the exchange of momentum and critical materials including dissolved oxygen and nutrients with surrounding water. Here we present a system capable of non-invasively measuring two-dimensional fluid velocity fields at sub-millimeter scales directly around benthic subjects in the ocean. This is achieved by applying micro-particle tracking velocimetry (μ PTV) underwater at long working distances. Specifically we combine an underwater microscopic imaging system with dark field illumination, rapid image acquisition, and particle tracking. *In situ* instrument deployments measured fluid velocity fields around individual coral polyps and provided direct observations of a viscous boundary layer at the coral's surface. The observational capabilities presented here offer a powerful new means for investigating basic physical processes that impact diverse marine organisms.

3.2 Introduction

A significant number of micro-scale processes influence the health of benthic marine ecosystems such as coral reefs and kelp forests. Recent advances in underwater microscopy¹ have afforded an important new way to observe such dynamics *in situ*. However, critical micro-scale interactions between benthic subjects and the surrounding fluid environment have remained difficult to study. Fluid motions at surface-water interfaces control the transfer of mass and momentum between the ocean and the benthic environment, yielding significant effects on physical, biological, and chemical systems²⁻⁴. Flows at sub-millimeter scales over benthic surfaces have important impacts on prey capture⁵, bio driven filter feeding⁶ and bio-mixing^{7,8}, as well as larval settlement and attachment⁹. Water motions here also regulate the rates at which benthic organisms are able to uptake dissolved solutes, including nutrients and oxygen, as well as to eliminate waste products. These rates of mass exchange may act as limiting factors for basic metabolic functions of ecologically important organism such as corals^{4,10-13}, kelps¹⁴ and seagrasses¹⁵, as well as microbes in marine sediments¹⁶⁻¹⁹. As a result, the fluid boundary layer has been referred to as “the most pervasive physical influence on the biology of many aquatic organisms”¹².

Mechanistically, a diffusive boundary layer (DBL) typically 100s of micrometers thick forms at the surfaces of benthic organisms and sediments. This slows mass transport and controls the flux of dissolved gasses and other solutes to and from the surrounding water. Critically, local water motions in turn control the DBL thickness, with increased flow producing a thinner DBL and thus greater mass transfer. Specifically, the DBL thickness scales with local shear stress²⁰⁻²³. Near surface velocity measurements thus offer a valuable tool for investigating mass transport to and from benthic organisms.

At typical distances of millimeters or less from benthic organisms and surfaces, viscous forces dominate momentum transfer; this creates a viscous boundary layer (VBL)²⁴⁻²⁶ in which average fluid velocities increase linearly with distance from the surface. Velocity profiles in the VBL provide a direct measurement of shear stress²⁷⁻²⁹ and offer a means for investigating DBL dynamics. However to date there have been only a limited number of velocity measurements within the VBL at the ocean benthos; this requires very fine-scale spatial resolution and measurements directly next to surfaces. Direct *in situ* measurements of the thin VBL have been performed over ocean sediments using profiling point sensors^{25,30,31} and profiling acoustic Doppler velocimeters (ADV)s^{32,33}. However, both of these approaches only provide one-dimensional velocity profiles and would likely encounter significant challenges if applied to observe the VBL around complex three-dimensional subjects such as branching corals^{33,34}.

Imaging velocimetry offers a potentially powerful approach for studying small-scale fluid dynamics at the surface-water interface over a variety of important marine subjects. Particle imaging velocimetry (PIV) and particle tracking velocimetry (PTV) provide quantitative visualizations of two-dimensional fluid velocity fields by tracking tracer particles in sequential images. Underwater PIV has been applied *in situ* at macro-scales to study boundary layer turbulence^{35,36}, flow over corals^{8,10,37,38}, organism swimming³⁹, and larval recruitment⁴⁰. Underwater PIV at the ocean the benthos has been performed with fields-of-view ranging from approximately 51 x 51 cm to 2.5 x 2.5 cm (with corresponding spatial resolutions of approximately 6.0 mm to 0.6 mm) at distances of several millimeters or more from surface-water interfaces^{35,37}. In the lab, bench-top μ PTV and μ PIV have been used to investigate sub-millimeter scale flows directly surrounding ocean specimen, including cilia generated vortices by coral polyps⁷ and starfish larvae⁴¹, as well as plankton swimming^{42,43} and organism feeding⁴⁴.

However these studies have been limited to observations in small, typically milli-fluidic, chambers. Alternatively, specialized long distance μ PTV has been developed in the lab to measure micro-scale fluid dynamics and shear stress over mechanical subjects in large scale turbulent flows^{28,29,45,46}; this technique has been applied to micro-structured surfaces^{47,48} and engines⁴⁹. Image velocimetry has thus proven to be powerful for both underwater macro-scale studies and lab microscopic observations. However, *in situ* image velocimetry measurements within the VBL at the ocean benthos have not been reported. This is likely because such measurements require sub-millimeter scale underwater imaging, precise focusing, and low surface light scattering.

Here we report a novel *in situ* technique capable of measuring fluid velocities within the VBL at the surfaces of benthic organisms, this is achieved through the design and application of a long distance underwater μ PTV system. Our system integrates μ PTV techniques with the recently developed Benthic Underwater Microscope (BUM)¹ to produce a new device that we label BUM-PTV. This diver-operated instrument can measure two-dimensional fluid velocity fields at sub-millimeter scales around spatially complex benthic organisms. In the following text we describe the system's design, instrument validation, and data processing. We then show *in situ* observations of micro-scale flows around individual coral polyps recorded in a coral reef.

3.3 Results

3.3.1 Instrument design

This work seeks to enable *in situ* observations of two-dimensional fluid dynamics, at sub-millimeter scales, directly around benthic marine organisms. These goals present several challenges: First, micro-scale images must be collected at the surface-water interface of benthic subjects *in situ*, in a minimally invasive manner. Next, images must observe only a thin sheet and resolve small suspended tracer particles. Additionally, frames must be recorded in rapid pairs and the water must contain particles following natural fluid motions. Finally, images must be processed to extract velocities from particle displacements. The instrument's design, as presented in Figure 3.1, addresses these challenges. With reference to Figure 3.1 (a,b) the system is operated underwater in the natural ocean environment, using long working distance lenses to minimize disturbances to organisms and natural water motions. Micro-scale imaging is achieved using the recently developed BUM (Fig. 3.1c), which is augmented with dark-field transmission illumination (Fig. 3.1d). While active particle seeding is used to increase natural particle densities, and image pairs are acquired in very rapid succession using a frame-straddling recording technique. As shown in Fig. 3.2, the individual particles are tracked between image pairs to quantify fluid velocities.

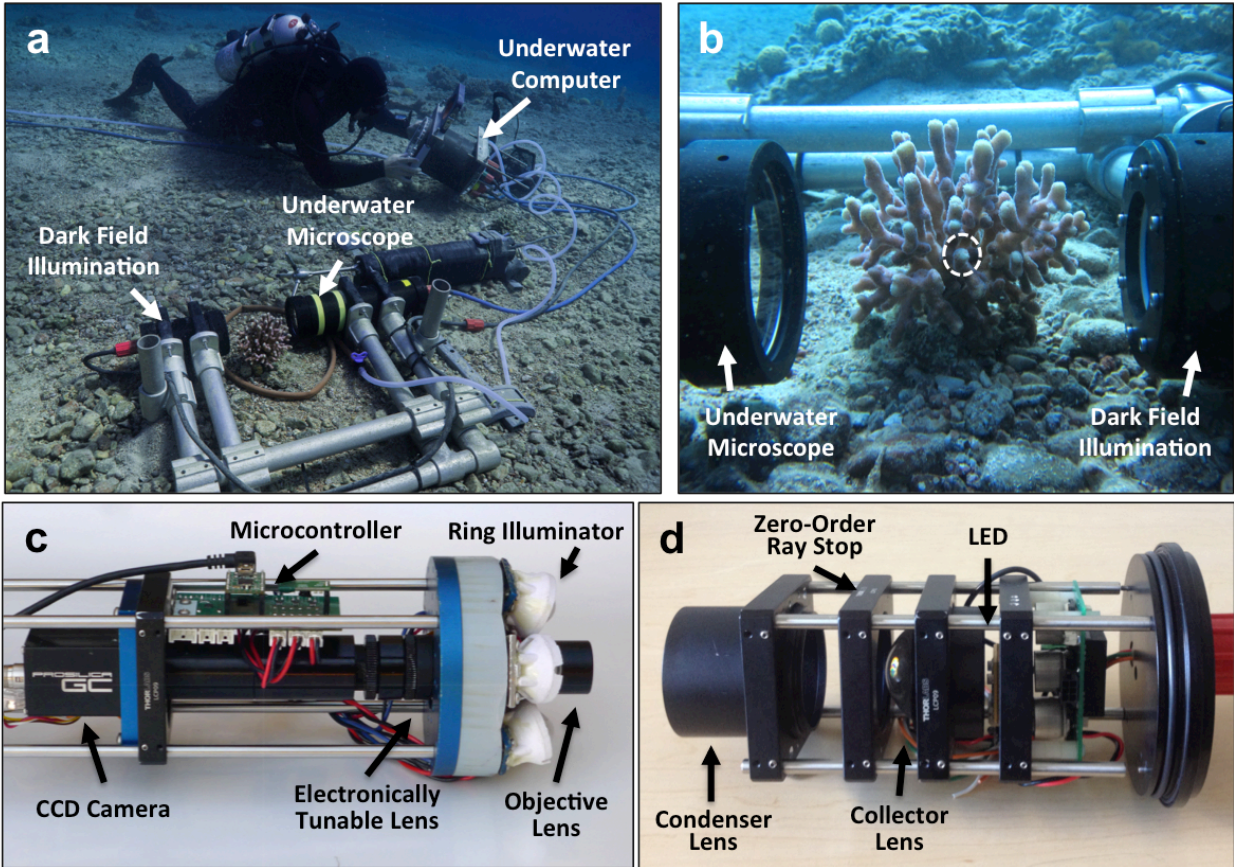


Figure 3.1 | Underwater μ PTV instrument design and operation. (a) *In situ* deployment of the μ PTV system around a small colony of the coral *Pocillopora spp.* Here a scientific diver is controlling the system underwater, an electronic cable was also routed back to shore allowing remote operation of the instrument once deployed. Tubing around the coral was used for active particle seeding. Also shown is an acoustic Doppler velocimeter that was placed next to the μ PTV system to observe nearby fluid motions. (b) Close up view of the system placed around the coral colony. Both housings are approximately 95 mm away from the imaged volume. The circle shows the approximate location of the imaged area. (c) Internal components of the underwater microscope imaging housing¹. (d) Internal components of the dark field illumination housing.

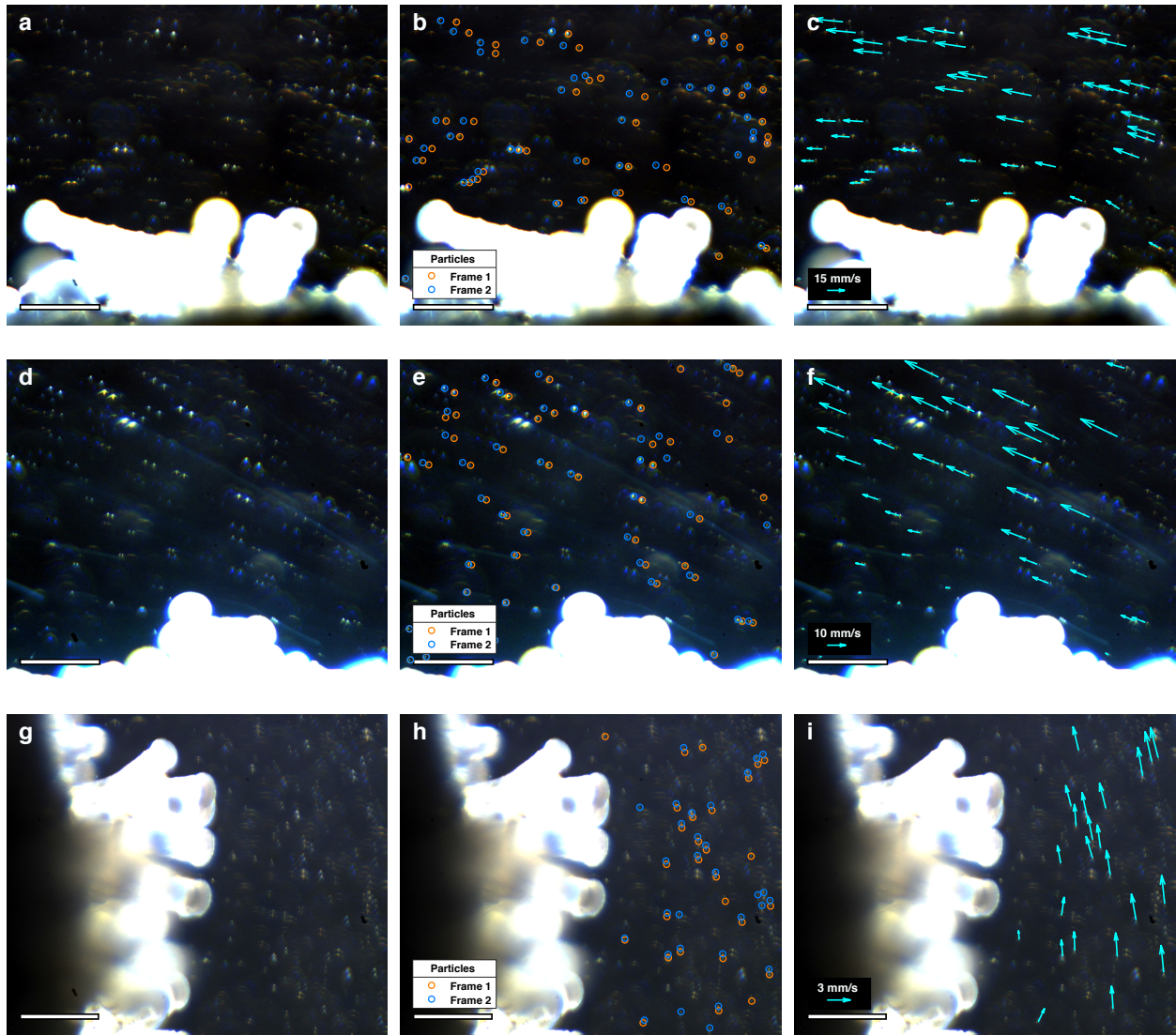


Figure 3.2 | Underwater μ PTV imaging and particle tracking. *In situ* images acquired of live coral polyps of *Pocillopora* spp. and particles in the surrounding water. Images shown are composites combining two sequential μ PTV frames in order to demonstrate particle movement between image pairs. Images recorded with active particle seeding. Vertical direction in images is up. (a,d,g) Image of a coral polyp and tracer particles. (b,e,h) Frame # 1 particles circled in orange, frame #2 particles circled in blue. (c,f,i) Vectors extracted from particle displacements. (Supplementary Videos 3.1-3.3 show the same coral polyps with particle tracking performed over 8 frame pairs.) Images in figure are strongly contrast stretched in order to make particles more visible. Scale bars 500 μ m.

The BUM-PTV imaging system uses a long working distance telecentric objective lens with a 3x magnification ($NA = 0.09$). This yields a field of view of approximately 2.5×2.1 mm (see Methods), while providing a working distance of 95 mm. The microscope objective additionally yields only a narrow in-focus volume, thereby optically segmenting a sheet of particles in the water for tracking⁵⁰. Here approximately 90% of particles detected by the μ PTV algorithm are within ± 500 μ m of the most in focus plane (details in Methods, Supplementary Fig. 3.1,3.2). The narrow in-focus plane enables the use of volume illumination, in contrast to macro-PIV which typically uses laser sheet illumination to segment the measurement plane³⁵⁻³⁷.

The imaging system additionally employs an electronically tunable lens (ETL)¹ (Fig. 3.1c), which provides a rapid and compact mechanism for adjusting the instrument's imaging focal length. This enables precise focusing on complex objects underwater, as well as the ability to rapidly scan the in-focus plane in order to observe fluid velocities at different focal depths.

To visualize tracer particles the optical system utilizes dark field transmission illumination. A hollow cone of light is focused onto the imaged volume and only forward scattered light is collected by the imaging lens (Supplementary Fig. 3.1). This technique produces enhanced contrast, which is critical for identifying small tracer particles, and also decreases noise from particles outside the imaging volume. The dark field setup was key to the success of the BUM-PTV system, allowing the visualization of particles directly adjacent to seafloor subjects. In comparison, previous underwater macro PIV systems using high powered laser sheets have experienced high light scattering at surfaces, which inhibited their ability to make observations close to subjects such as coral polyps³⁷. Here, light is provided by a high intensity pulsed LED⁵¹. The LED light is first collimated, the zero order (central) rays are blocked, and the light is then focused onto the imaged volume. This design enabled very short

illumination pulses of 100 μs or less which are needed to produce low particle blur. Additionally, the illuminator provided a working distance of approximately 95 mm, minimizing the effects of the illumination housing on natural water motions in the imaging volume.

Previous macro-scale underwater PIV systems have relied on naturally occurring ambient particles as tracers^{10,35,36}. However, the imaging volume provided by BUM-PTV is much smaller, and the number of naturally occurring particles imaged by the system is low. We therefore actively seed the water with inert, neutrally buoyant 10 μm diameter hollow glass spheres (HGS) that act as tracer particles (Supplementary Fig. 3.3, details in Methods). Vector field density can also be increased by combining velocity measurements from several consecutive image pairs.

The BUM-PTV instrument is controlled and powered by a custom underwater computer¹. This allows the entire system to be deployed *in situ* to image subjects of interest in a minimally invasive manner under natural conditions. During diver deployments the instrument can operate completely untethered, alternatively it can be connected to a shore cable to enable remote power and control after instrument positioning (Supplementary Fig 3.4, 3.5). Once positioned the imaging system can acquire frame pairs with inter-frame intervals as short as 50 μs (see Methods) in order to constrain particle displacement.

Following data acquisition, images pairs were processed to extract velocity vectors using standard PTV analysis⁵² (Fig. 3.2, Supplementary Video 3.1-3.3, details in Methods). Images were collected in regions of high fluid velocity shear, directly adjacent to surfaces, with relatively low particle concentrations (even with active seeding). Under these conditions μPTV , which tracks individual particles, has been shown to have increased accuracy and performance over μPIV , which performs cross correlations^{45,46}. Work here follows the approaches of previous

μPTV efforts: Images are first filtered to remove noise. In-focus particles are then identified and their locations determined⁵³. Finally, particles are matched between image pairs and particle displacements are used to calculate velocity vectors⁵². Ensemble averaging of velocities can additionally be performed on time series data to increase spatial resolution, and build average two-dimensional velocity fields⁵⁴.

3.3.2 Lab flume experiments and vector analysis

The BUM-PTV's performance was validated by measuring fluid velocities at the surface of a flat knife-edged plate in a recirculating lab flume (details in Methods, Supplementary Fig. 3.6) and comparing these velocities with theory. Time series recordings were collected directly above the flat plate, over 25 sec time periods at a rate of 4 frame pairs per second. This was performed at several different flume flow speeds. A time-averaged, velocity profile as a function of distance from the wall was then determined for each time series data set (Fig. 3.3a-d). First vectors were grouped as a function of distance from the plate surface in 100 μm bins. Next, a mean horizontal, stream-wise, velocity was calculated for each bin. Then, an ordinary least squares linear fit was applied to the average bin velocities in the approximately linear portion of the velocity profile ($y^+ < 8$) closest to the surface. Finally, the coefficient of determination (r^2) and standard error of the gradient (SE) could be calculated for the linear fit. This analysis approach follows previous work e.g. Li²⁹, Hutchins and Choi⁵⁵.

The near-surface average velocity profiles closely fit a linear slope (Fig. 3.3a-d); this indicates that the BUM-PTV system is observing fluid motions within a VBL at the plate's surface. The gradient of the velocity profile within the VBL can be used to directly calculate the mean wall shear stress, $\overline{\tau_w} = \mu \frac{\partial \bar{u}}{\partial n} \approx \mu \frac{d\bar{u}}{dn}$ (where $\mu = \nu \rho$ is the dynamic viscosity of the fluid),

and the friction velocity, $u_* = (\overline{\tau_w} / \rho)^{1/2}$.^{28,29,45,48} The friction velocity can then be used as a scaling factor to transform the average velocity profile into non-dimensional wall units, $y^+ = y u_* / \nu$, and velocities, $u^+ = \bar{u} / u_*$. Figure 3.3e compares these non-dimensional velocity profiles to the theoretical ‘law of the wall’ which describes a universal mean velocity profile for turbulent flow over a flat plate: $u^+ = y^+$ for $y^+ < 5$ (the viscous layer), and $u^+ = \frac{1}{\kappa} \log(y^+) + B$ for $y^+ > 30$ (the log layer). In the log layer $\kappa=0.41$ is the von Karmen coefficient and typically $B=5$, our measurements fit to a slightly higher value of $B=6.5$, likely due to surface roughness or a pressure gradient⁵⁶. Overall, the non-dimensionalized BUM-PTV measurements closely match the law of the wall theory (Fig. 3.3e). Additionally, it can be seen that as the flume speed increased the average velocity gradient and shear stress increased and the linear VBL region became thinner.

Instantaneous measurements of shear stress were also calculated by applying a linear fit to the near surface vectors of each individual image pair. For turbulent flow over a flat plate, the instantaneous wall shear stress naturally fluctuates, and controlled experiments have reported values of normalized fluctuation intensity, $\tau_{w,rms}^+ = \frac{(\tau_w - \overline{\tau_w})_{rms}}{\overline{\tau_w}}$, ranging from 0.28 to 0.43.^{29,57,58} Here, the BUM-PTV system measured a value of $\tau_{w,rms}^+$ of 0.28 at a flume speed of approximately 90 mm/s. Additionally the probability density function (PDF) of the normalized instantaneous shear stress ($\frac{\tau_w}{\overline{\tau_w}}$) measured here approximates a log-normal distribution, this matches the findings of previous studies^{57,58} (Supplementary Fig. 3.7).

These laboratory results demonstrate the BUM-PTV system’s ability to: 1) observe sub-millimeter scale flows over surfaces, 2) resolve flows inside the VBL and use the velocity profile to determine wall shear stress, 3) operate over a range of speeds relevant to ocean flows, and 4) resolve instantaneous shear stress fluctuations.

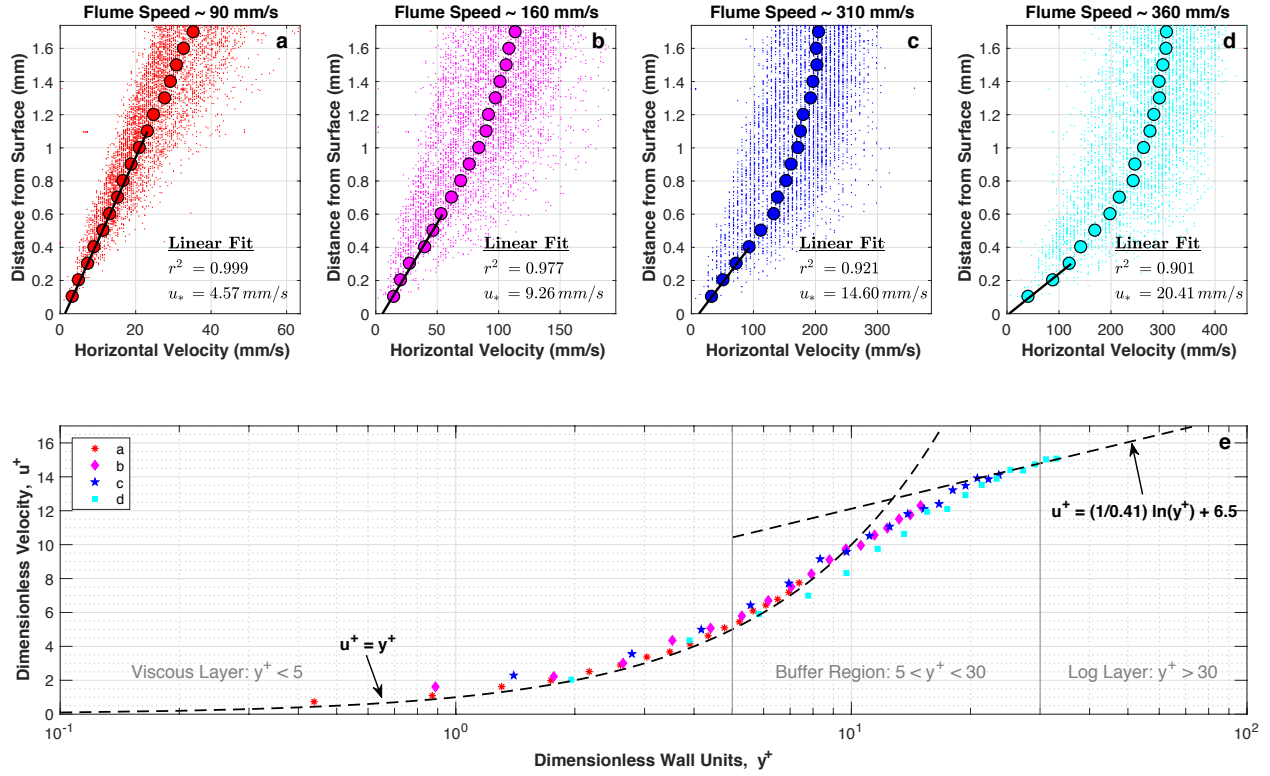


Figure 3.3 | μ PTV measurements over a flat plate in a lab flume. (a-d) Horizontal velocity as a function of height above the flat plate, measurements performed at several flume speeds. Each figure was produced from 100 μ PTV frame pairs (25 sec time period, images recorded at 4 frame pairs per second). Small dots in scatter plots show horizontal velocities of all individual tracked particles. Large circles show averages of horizontal velocities binned in 100 μ m intervals from surface. A least squares fit was applied to the approximately linear portion of the average velocity profile ($y^+ < 8$) for each data set, shown by the black line. The linear velocity gradient was then used to determine a friction velocity (u_*) for each flow speed (e) Average velocity profiles from a-d were non-dimensionalized and compared to ideal law of the wall theory. For each flume speed the corresponding friction velocity was used to transform u and y into non-dimensional wall units (y^+) and velocities (u^+). Non-dimensional μ PTV results were then plotted over a universal law of the wall profile. The law of the wall predicts a linear region at wall distance $y^+ < 5$ where viscous forces dominate, followed by a transitional or buffer region, and a logarithmic region at wall distances $y^+ > 30$ where turbulent forces dominate.

3.3.3 *In situ* coral reef deployment

The *in situ* capabilities and potential of the BUM-PTV instrument were demonstrated through field deployments conducted at approximately 5 m depth in the coral reef offshore of the Interuniversity Institute for Marine Sciences (IUI) in the northern Red Sea, Eilat, Israel. During field operations unique micro-scale velocity measurements were collected at the surface of a live, naturally occurring coral colony of the genus *Pocillopora*. The BUM-PTV system was deployed by scuba divers, and positioned to image the coral's outer branches (Fig. 3.1a,b). Underwater μ PTV observations were collected of polyps on several different branches and under different flow conditions. Coral polyps in the imaging field of view remained extended during recordings, suggesting minimal instrument disturbance to the coral during normal operations. The BUM-PTV was controlled both underwater by divers and from shore after deployments using a long interface cable.

In situ BUM-PTV images show both the coral polyps (~1 mm in diameter) and tracer particles in the directly adjacent water (Fig. 3.2). Particles were tracked between image pairs to measure fields of velocity vectors (Fig. 3.2b,c,e,f, Supplemental Video 3.1-3.3). *In situ* fluid velocities were measured at speeds ranging from less than 1 mm/s to greater than 20 mm/s. Dark field illumination pulses of 100 μ s or less were used, resulting in approximately one pixel of particle movement during illumination for typical flows. Image pair separation times on the order of 1-10 ms were used to provide particle displacements of tens of micrometers. With active seeding the system was capable of recording typical fields of 20-50 vectors per frame pair.

Time series μ PTV recordings provide a means to measure a large number of vectors, and time averaging can be used to increase spatial velocity resolution. Here time averaged velocity fields surrounding coral polyps were determined for short time series recordings of 2 seconds

each (8 frame pairs) as shown in Fig. 3.4 a,c,e. First all vectors recorded during the time intervals were combined (Supplemental Fig. 3.8 a,d,g). An average velocity at each two-dimensional position in the image was then calculated based on a Gaussian weighted average of all vectors within a 400 μm radius, following the technique described in Aguera⁵⁴ (Fig. 3.4 a,c,e). Time averaged velocity fields surrounding the coral polyps demonstrate a strong velocity gradient, with flow speeds increasing with distance from the surface.

Next, the velocity profile as a function of distance from the coral surface was investigated (Fig. 3.4 b,d,f). The position of the coral's surface in the image was first determined using intensity thresholding. The minimum distance from each vector to the surface was then calculated for the time series. The velocity component of each vector parallel to the surface was also calculated, defined as the velocity perpendicular to the local surface normal vector (Supplementary Fig. 3.8). Similar analysis approaches have been applied to *in vivo* flows within blood vessels^{59,60}. All vectors were then combined to calculate an average velocity profile, following the methods described in the lab flume data section: mean velocities were calculated in 100 μm intervals from the surface, a linear fit using an ordinary least squares approach was applied to these mean velocities, wall shear stress and friction velocity were then calculated based on the linear fit (values reported in Fig. 3.4 b,d,f).

Underwater BUM-PTV measurements reveal an approximate linear average velocity profile directly adjacent to the coral. These measurements provide direct evidence of the existence of a VBL at the coral's surface *in situ*. This VBL was observed under varying coral polyp geometries and flow conditions. The velocity gradient in the VBL provides a direct measure of wall shear stress and can be used to calculate friction velocity. However, note that the BUM-PTV system does not measure out-of-plane fluid motions. As a result measurements of

shear stress and friction velocity are lower bounds and they may be greater if there is significant additional out-of-plane flow. Additionally it can be seen that the average velocity gradients intersect the surface at slightly positive velocities, this is likely due to the finite thickness of the particle detection plane and some particles being detected in front of or behind the polyps protruding from the coral branch. Nonetheless the velocity gradients exhibit very high coefficient of determination (r^2) values, indicating the averaged measured velocities closely fit a linear profile.

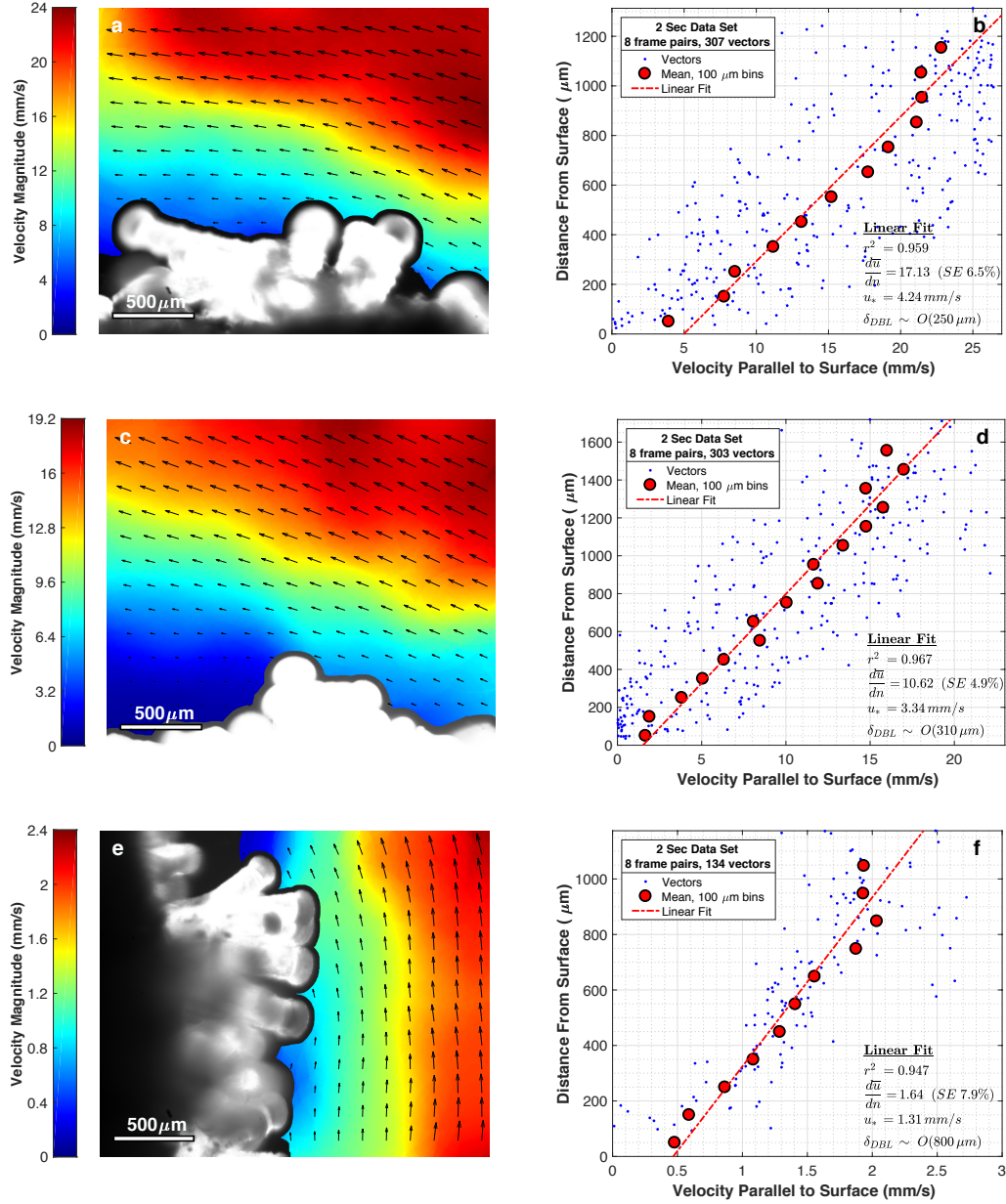


Figure 3.4 | Time averaged μ PTV measurements surrounding *in situ* coral polyps. Average velocities in each figure were calculated from 8 frame pairs recorded over a 2 sec time period. (a,c,e) Two-dimensional time-averaged velocity fields; an average velocity at each point was calculated using a Gaussian weighted average of all vectors within a 400 μm radius over the entire time series. (b,d,f) Velocity parallel to the coral's surface (u) as a function of distance from the coral surface (n). Mean velocity determined every 100 μm , and a linear fit was applied to the average profile. Figure text reports the linear fit coefficient of determination (r^2), the gradient ($\frac{d\bar{u}}{dn}$), and the standard error of the gradient slope fit (SE). The velocity gradient was used to calculate friction velocity (u_*) and approximate the DBL thickness (δ_{DBL}) using simple scaling based on the Schmidt number. Legends report the number of image pairs and vectors used to create each figure. (Supplementary Videos 3.1-3.3 show particle tracking for each data set; Supplementary Fig. 3.8 shows all vectors used to create figures.) Scale bars 500 μm .

Velocity measurements of the VBL also provide a means to estimate the thickness of the chemical DBL, which is hydrodynamically controlled^{2,18,20,21}. This scaling can be performed using the Schmidt number, which is a ratio of momentum diffusion to molecular diffusion ($Sc = \frac{\nu}{D}$, ν is kinematic viscosity and D is molecular diffusion)^{2,20,21}. Mass transfer theory based on steady flow over a flat surface predicts: $\delta_{DBL} \approx \delta_{VBL} (Sc^{-\frac{1}{3}}) \approx 10 \frac{\nu}{u_*} (Sc^{-\frac{1}{3}})$.²⁰⁻²² Analysis using Schmidt number scaling has been applied to a variety of ocean subjects^{2,4,14} and experimentally tested over marine sediments^{21,22} (details in Methods). Numerical simulations of non-steady flows over corals have shown that this scaling relationship may be more complex for rough coral surfaces in the ocean; but significantly, local shear stress still acted as a proxy for local mass transfer²³.

Here we calculate an approximate DBL thickness by applying simple scaling using the Schmidt number and the friction velocity as determined from μ PTV velocity gradient measurements: $\delta_{DBL} \sim 10 \frac{\nu}{u_*} Sc^{-\frac{1}{3}}$ (with $Sc = 1,000$, details in Methods)²⁰ (Fig.4 b,d,e). Given the complexity of the system this calculation is considered to be an order-of-magnitude approximation. Previous chemical measurements at the surfaces of corals^{11,61} and marine sediments^{16,18} using profiling microsensors have shown typical DBL thicknesses of 100 μ m to 1mm. Significantly, the approximations performed here using scaling relationships fall within this expected range. μ PTV velocity measurements are powerful as they provide a means to investigate the fine-scale spatiotemporal fluid dynamics controlling the local chemical microenvironment and flux rates; as well as how these fluid dynamics vary with time, location, and ocean conditions.

3.4 Discussion

Here we presented the design of a novel *in situ* system, BUM-PTV, capable of measuring two-dimensional velocity fields at sub-millimeter scales around complex subjects in the ocean. As compared to existing velocimetry techniques used to observe the benthic ocean VBL^{25,31,32,34}, μ PTV uniquely provides whole-field velocity measurements at micro-scales and can be applied directly at surfaces of complex specimen such as branching corals. Controlled lab experiments validated the ability of the BUM-PTV system to measure velocity profiles and wall shear stresses at a range of flow speeds corresponding to common velocities in benthic marine environments. Underwater deployments then demonstrated the system's ability to observe micro-scale fluid motions directly adjacent to coral polyps *in situ*. These observations uniquely show the presence of a linear velocity gradient and VBL at a coral's surface in a natural reef environment. The results of this work provide an important new observational capability that enables the study of fluid dynamics impacting a variety of marine organisms.

Building on the techniques developed here, ongoing analysis of *in situ* μ PTV data is being used to investigate spatiotemporal dynamics of the physical microenvironment at the coral's surface. Flows on reefs as well as other marine environments are often non-stationary and changes in the outer flow likely drive changes in the VBL and DBL¹⁸. Oscillatory wave motions may have a significant effect on near surface fluid dynamics and mixing²³. Additionally, work on sediments has suggested that the benthic DBL may be periodically mixed by turbulent "sweep-ejection" events⁶². μ PTV observations offer potential to provide new insight on these important temporal fluctuations.

Benthic subjects such as corals also have rough surfaces and intricate three-dimensional structure. Corals have been hypothesized to potentially modulate the flow environment at their

surface through polyp extension and contraction³⁷, and have been shown to generate cilia driven currents in the lab⁷. Small-scale surface roughness may have varying influences on mass transfer in a variety of benthic systems^{16,17}. Underwater μ PTV offers a tool uniquely capable of examining two-dimensional fluid dynamic processes over complex surfaces at fine scales. Micro-scale velocity measurements also enable potential *in situ* investigations into forces important to larval settlement⁹, and biologically driven currents⁶⁻⁸. Similarly, fluid velocity measurements in the VBL at the seafloor may provide important insights on physical processes such as sediment transport and re-suspension^{63,64}, and wave-current boundary layer dynamics^{63,65,66}. Finally, μ PTV observations of organisms can also be collected in lab flumes, which offer a controlled environment and an intermediate step between milli-fluidic chambers and *in situ* measurements.

One significant limitation of the μ PTV system, common to all two-component imaging velocimetry systems, is the inability to measure the out-of-plane velocities perpendicular to the imaged plane. True particle velocities may be greater than the measured values if there is significant out-of-plane motion; as a result, the system can only establish a minimum bound on the average velocity gradient and shear stress. Considering this constraint, the system should be positioned such that the measured plane is parallel with the strongest water motion. Several approaches offer potential for resolving the out-of-plane velocity component in future underwater μ PTV systems including focal scanning, coded aperture, depth from defocus, tomography, and holography⁶⁷. Another measurement consideration is the number of vectors per image, this can be increased by using greater particle seeding concentrations or by tuning the tracking algorithm to detect particles in a thicker plane. Flow restoration algorithms⁶⁸ also offer potential to fill in sparse vector fields.

Potential impacts of the instrument on natural water motions must also be considered. Imaging velocimetry is a non-intrusive technique and the system described here leaves approximately 95 mm of space from the measured volume to each optical housing. Future underwater μ PTV systems may be able to achieve longer working distances of several hundred millimeters, while maintaining high spatial resolution, by using a setup similar to existing long distance μ PTV lab systems^{28,29,48}. However, these lenses have a lower numerical aperture and thus require laser sheet illumination, so caution would need to be taken regarding surface light scattering as well as alignment.

The interactions between marine organisms and their physical environment are especially relevant during this period of global change. Fluid motions have been shown to have important impacts on the ability of corals and other marine organisms to cope with stressors such as ocean warming⁶⁹ and acidification⁷⁰. Water motions modulating the DBL affect coral photosynthesis^{8,10} and their ability to eliminate reactive oxygen species which cause coral bleaching during high temperature events⁶⁹. Small-scale fluid dynamics can also have strong influences on the pH directly at the coral's surface⁷⁰.

Micro-scale fluid motions at the surfaces benthic organisms and sediments impact a wide variety of basic and significant environmental processes². These fluid motions are driven by a complex combination of currents, waves, eddies, and surface structure that is difficult to replicate in the lab. By merging bench top and field techniques we are able to bring the lab into the ocean, providing a new means to study this important microenvironment under natural conditions.

3.5 Methods

3.5.1 BUM-PTV optics

The imaging system uses a long working distance microscopic objective lens (Mitutoyo ML X 3, NA = 0.09) to provide optical magnification and resolving power. A shape changing ETL (Optotune EL-10-30-C, $f = +80$ to $+200$ mm) enables rapid, electronically controlled, focus adjustment. The ETL is paired with a plano-concave offset lens (Thorlabs LC1120-A, diameter = 25.4 mm, $f = -100.0$ mm), which slightly increases the system's working distance. Images are recorded with a machine vision color CCD camera (Prosilica GC2450, 2,448 x 2,050 pixels, 3.45 μm pixel pitch, maximum frame rate of 15 fps at full resolution, global shutter, interline-transfer CCD architecture). The imaging housing also includes a reflectance illumination ring consisting of six LEDs (LED Engin LZ1). This was not used during normal μPTV image acquisition, when light was provided by dark field transmission illumination.

The dark field illumination unit uses a pulsed a high current LED (Lumis CBT) as a light source⁵¹. The LED light is collimated by a plano-convex collector lens (Thorlabs ACL5040, diameter = 50.0 mm, $f = 40.0$ mm). The central portion of the collimated rays are then blocked by an optical stop (custom laser cut, diameter = 32 mm). Finally, the light is focused with a spherical plano-convex condenser lens (Thorlabs LA1050, diameter = 50.8 mm, $f = 100.0$ mm). Light leaves the illumination housing as a hollow cone focused at the imaging plane, and only forward scattered light is captured by the imaging lens. Optical components are held in place using Thorlabs opto-mechanical cage elements (Thorlabs LCP09, ER6E, SM2L05).

3.5.2 BUM-PTV electronics

A micro-controller inside the imaging unit (Teensy 2.0 running Arduino Code), mounted on a custom PCB, controls and synchronizes the camera shutter, ETL, and the dark-field LED trigger. A trigger line runs from the microcontroller in the imaging housing through the underwater computer to the dark-field illumination housing. The dark-field illumination housing also contains its own custom PCB. This receives a trigger signal and pulses the LED with a circuit that includes: a signal opto-isolator, MOSFET driver, MOSFET power switch, voltage converter, and bank of three capacitors (4700 μ F). The LED trigger circuit is similar to that described by Willert et. al. 2010⁵¹. A custom underwater computer¹ is used to provide a scientific diver control of the system, to power the camera and illumination, and to run imaging protocols. The underwater computer housing includes a 5 inch LCD screen, eight piezo-electric buttons (Baran Advanced Technologies), compact computer (Intel NUC), 500 Gb solid-state hard drive, and four Li-Ion batteries (Ocean Server) allowing approximately 8 hours of untethered system operation. A graphical user interface provides a diver full control of the camera and illumination as well a live view of acquired images; software was based on the camera's SDK and written in C++. SubConn cables and connectors provide electrical connections between the imaging, illumination, and underwater computer housings.

After initial deployment and positioning by diver, the system can optionally be connected to shore via a power and data cable (100 m long, Subconn). This provides a shore station with a live image feed and instrument control as well as supplying continuous power to the underwater μ PTV system and allowing data transfer (Supplementary Fig. 3.7). With this configuration the system was operated periodically from shore for several days at a time without need for repeated dives.

3.5.3 BUM-PTV mechanical design

Underwater housings for the imaging unit (39.5 cm length x 12.2 cm diameter), illumination unit (21 cm length x 12.2 cm diameter), and underwater computer (30.6 cm length x 21.9 cm diameter) were machined from 6061-T6 aluminum cylinders. Imaging ports were machined from Spartech Polycast Super Abrasion Resistant acrylic. The imaging and underwater computer housings were pressure tested to a depth of 30 m, which is sufficient for normal SCUBA operations. Significantly greater pressure ratings can be achieved by using thicker end caps. Related micro-scale imaging systems developed in our lab have been pressure rated for depths greater than 300 m.

The imaging and illumination housings are held in alignment by an aluminum frame constructed from Speed-Rail (Hollaender). The frame could be loosened to make small adjustments during alignment, and then tightened for underwater deployment. This offered a modular system that could be easily deconstructed and reconfigured. The dark field illumination requires very precise alignment with the microscopic imaging housing. Prior to *in situ* deployments the μ PTV system's optical housings were aligned while submerged in a small pool.

3.5.4 Particle seeding

Hollow glass spheres (Potters Industries Spherical® 110P8 Hollow Glass Spheres, borosilicate glass, average diameter = 10 μ m, density = 1.1 g/cm³) were used as tracer particles and actively seeded into the water during *in situ* experiments. These are commonly used in lab PIV experiments and have several advantages for this application. The borosilicate glass material is highly inert and of similar chemical composition to natural sand. Particles are close to neutrally buoyant and of small size such that they follow fluid motions. The particle material and

size is efficient for forward light scattering, allowing particle visualization. They are relatively inexpensive, available in bulk, and a small volume is needed to seed a large amount of water.

Particles were mixed with seawater in a plastic reservoir tank at the end of the IUI pier (Supplementary Fig. 3.3). A very small volume of particles was needed to produce water of sufficient particle density (typically on the order of 1 cm^3 of particles or less per liter of water). In the future, if images with higher particle densities are needed, the seeding water could be mixed with significantly increased particle concentrations. Plastic tubing funneled this water-particle mixture from the pier reservoir to the underwater μ PTV system, using gravity driven flow. The water-particle mixture was dispersed into the seawater by a rubber irrigation tubing looped in a circle (approximately 30 cm in diameter) around the coral subject. Several hundred small needle hole pricks in the tubing facing outwards away from the center of the loop were used to slowly release the mixture into the ambient water (at a rate on the order of a few liters per min) in a distributed manner with minimally disturbance to natural fluid motions (Supplementary Fig. 3.4). Having the tubing looped in a circle ensured that particles would reach the imaged volume no matter the direction of the ambient currents.

While active particle seeding was used here, coral reefs are oligotrophic environments with low natural particle densities. Other marine environments or locations may have sufficiently higher natural particle densities such that active tracer particle seeding is not needed.

3.5.5 Image acquisition timing

Sequential images with very small time separations are needed to achieve small particle displacements between frames for particle tracking. A "frame straddling" technique is utilized to meet this requirement, in which the camera records two long but nearly immediately sequential

exposures (this is possible due to the camera's interline CCD architecture). The period of time during which the particles are recorded is controlled by the pulsed illumination, which is flashed near the end of the first exposure and beginning of second exposure (timing diagram in Supplementary Fig. 3.1). Using this method the system was capable of achieving inter-frame times of $<50\ \mu\text{s}$, allowing potential observations in high flow environments. However, under typical field conditions experienced during this study inter-pulse times of 1-10 ms were used. Due to the required long duration exposures (approximately 0.125 sec for images recorded at 8 frames per second), *in situ* studies were conducted primarily at night in order to reduce ambient light noise. Illumination timing accuracy was tested in the lab using an oscilloscope and photodiode. This showed the pulse timing was accurate to $\pm 6\ \mu\text{s}$, sufficient for the typical inter-pulse durations of several milliseconds used *in situ*.

3.5.6 Image processing

All image processing was performed in Matlab, using code based on existing algorithms. First, image intensities are normalized between frame pairs to account for potential variations in LED intensity. Bandpass filtering is then used to eliminate noise and an edge detector applied to emphasize particles. The coral's surface is determined using image intensity thresholding and morphologic processing, and this region is masked out. Particles are then identified using an algorithm based on the IDL tracker⁵³, and their location determined to sub-pixel accuracy using a Gaussian fit to the particle image⁵³. (Note, that some particles are clearly visible but not detected, this is likely because they are slightly out of focus. The tracking algorithm can be adjusted to detect more particles, but velocities will be measured over a thicker volume.) Next, particles are matched between image pairs using a non-iterative approach that is constrained by the potential

matches of neighboring particles, based on Fuchs et. al.⁵². Velocity vectors are calculated for all matched particles. Global filtering based on velocity magnitude and direction was then used to eliminate likely spurious vectors as a means quality control. For μ PTV conducted in the flume a local filtering approach was used for quality control rather than the global approach due to very strong shear at high flow speeds.

3.5.7 Optical calibration

The μ PTV system's field-of-view (FOV) and scan range were calibrated in the lab while the instrument was setup around the flume tank. A micromanipulator mounted on the top of the flume held a Ronchi ruling (20 line pairs/millimeter) submerged in the flume within the μ PTV imaged volume. In the optical system the ETL is positioned just after the objective lens, this causes the FOV to change slightly over the scan range. The system was measured to have a FOV of 2.52 x 2.11 mm at the central ETL position (each CCD pixel imaging an approximately 1.0 x 1.0 μ m area). This FOV changes slightly from a minimum of 2.32 x 1.94 mm to a maximum of 2.66 x 2.23 mm over the scan range (a total change in the vertical and horizontal dimensions of approximately 14%). Additionally, the ETL was measured to have a scanning range in the z-direction of 18 mm.

The microscope objective lens yields only a thin in-focus volume; and this is relied upon to segment a thin sheet of particles for velocity measurements⁵⁰. The measurement plane width (MPW) of the BUM-PTV velocity measurements, was calibrated in the lab. HGS tracer particles were sandwiched between two microscope slides. This particle slide was then imaged with the BUM-PTV setup and a micromanipulator was used to translate the slide in the z-axis while the ETL focus was held constant. This produced a series of images in which the particles go from

out-of-focus to in-focus to out-of-focus (Supplementary Fig. 3.2). The particle identification algorithm was then applied to each plane in this series of images. The number of detected particles as a function of particle z-position approximates a Gaussian distribution with a standard deviation of approximately $\pm 300 \mu\text{m}$ (Supplementary Fig. 3.2). We therefore expect approximately 67% of detected particles to occur within $\pm 300 \mu\text{m}$ from the most in-focus plane, and approximately 90% of detected particles occurred within $\pm 500 \mu\text{m}$ from the most in-focus plane.

It is important to note that the thickness of the MPW is a function of the instrument's optics as well as the particle detection algorithm applied and the tracer particles. It is thus possible to change the MPW by adjusting the particle identification algorithm. During testing, the MPW for 90% of detected particles could be varied from roughly $\pm 300 \mu\text{m}$ to $\pm 1000 \mu\text{m}$ by adjusting the particle identification algorithm, with a thicker MPW detecting significantly more total particles. The same detection settings (90% of particles detected within $\pm 500 \mu\text{m}$ of in-focus plane) were used for all processing shown here.

3.5.8 Flume testing

Lab work was conducted in a flume based on the commonly used Vogel design (Supplementary Fig. 3.6). The flume's channel was approximately 122 cm long x 15 cm wide x 20 cm deep and constructed from clear acrylic. The flume was filled to a depth of 15 cm with filtered seawater and seeded with HGS. Two flow collimators (9.5 cm long, spaced 4 cm apart) constructed from drinking straws were placed at the start of the flume to reduce turbulent fluid motions. The flume was driven by a rotating propeller in the return flow (powered by a 1/9 hp motor), and was capable of generating average flume water speeds ranging from approximately

50 mm/s to 400 mm/s. The BUM-PTV system was positioned around flume, with the imaging housing pressed against the flume wall. CCD pixel binning (2x2 pixels) was used at high flow speeds to allow illumination pulses as short as 30 μ s in order to constrain particle blur. Flume velocities were calibrated by measuring flow in the center of the channel with the μ PTV system while several different power inputs were applied to the propeller motor. (Flume speed calibrations were performed without the knife-edged plate in the tank).

A knife-edged plate machined from Delrin, (51 cm long, 15 cm wide, 0.8cm thick) was mounted in the middle of the flume parallel to the flume floor. μ PTV measurements were made at the surface of the plate in the center of the tank, approximately 35 cm downstream from the plate's leading edge. The μ PTV measurements match relatively well with previous experiments and law of the wall theory. The instantaneous shear stress normalized fluctuation intensity ($\tau_{w,rms}^+$) was measured to have value of 0.28, within the range of previous measurements but relatively low. This small value may be due to the low flume speed of approximately 90 mm/s, as well as potentially due to effects of spatial averaging over the imaged volume^{57,58}.

3.5.9 *In situ* deployments & data selection

Fieldwork was conducted on a relatively flat and sandy seabed at a depth of approximately 5 m, roughly 30 m offshore of the Interuniversity Institute for Marine Sciences (Supplementary Fig. 3.4). The BUM-PTV system was deployed from shore by a team of two scientific divers and carefully placed around a small, relatively isolated, coral colony of *Pocillopora spp.* The sandy bottom enabled fine adjustments of the position of the system's frame in order to center the imaging field-of-view on a coral polyp and the surrounding water. (The soft bottom was critical for this fine-scale positioning; in the future a fine position

adjustment mechanism could be integrated into the frame.) Precise focusing in the z-axis was then achieved using the ETL. During underwater image acquisition, divers took caution not to be upstream of flow or move significantly, so as to avoid influencing natural water motions. After system deployment μ PTV image acquisition and particle seeding could be controlled from a shore station setup on the nearby IUI pier (Supplementary Fig. 3.5).

In situ μ PTV measurements used to create Figure 2 and 4 were selected during time periods when flow was relatively steady and parallel to coral's surface. In some μ PTV data sets flows were impinging on the coral, although in the majority of observations flow was primarily parallel to the surface. Images were additionally selected during periods of time in which there were relatively high particle concentrations, although there were some time periods with at least twice the number of particles as shown here. In the future greater particle concentrations may be achieved by adding more particles to the seeding mixture or by using a thicker MPW as controlled by the particle detection algorithm.

3.5.10 Diffusion boundary layer scaling

At scales of millimeters from the seafloor and benthic organisms a viscous boundary layer (VBL) forms, in which viscous forces dominant momentum transfer²⁴⁻²⁶. Average fluid velocities here increase linearly from the benthos, and velocity profiles in the VBL can provide a direct measurement of shear stress²⁷⁻²⁹. Within the VBL turbulent fluid mixing eventually become sufficiently small that solute flux is dominated by molecular diffusion. This creates a diffusion boundary layer (DBL), typically hundreds of micrometers thick at the surface of the seafloor and benthic organisms. In the DBL there is a linear gradient in dissolved solutes and gases between the surface and the surrounding water^{16,24}. The DBL inhibits the flux of dissolved

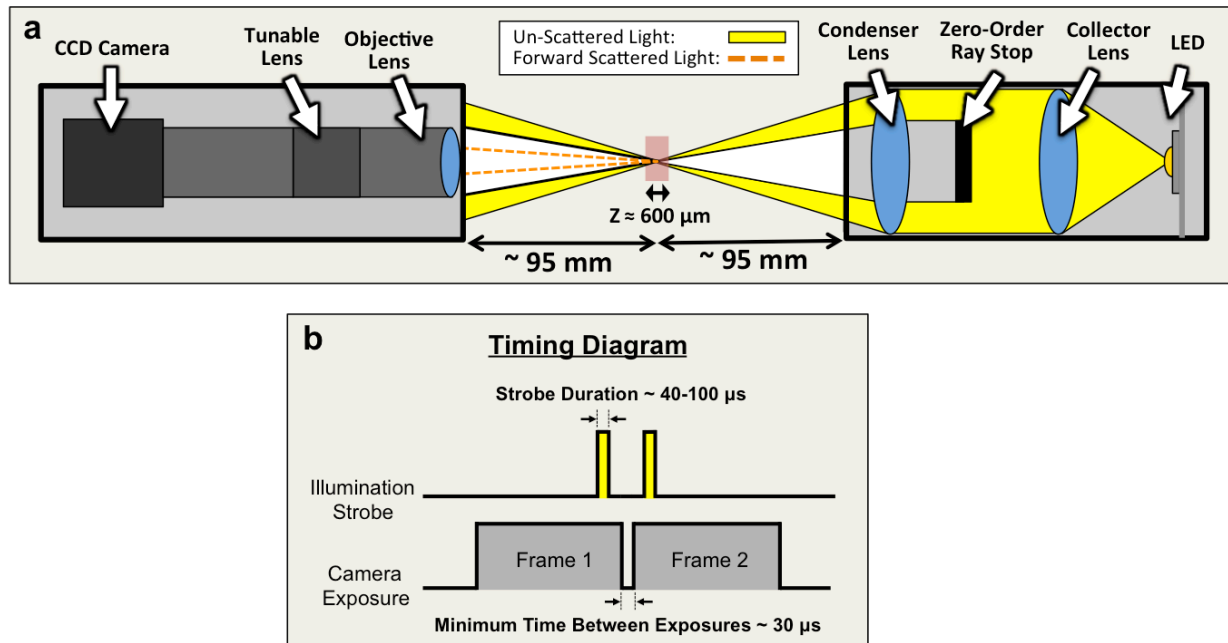
solutes such as oxygen, carbon dioxide, and nutrients; and may have major impacts on organisms. Significantly, the thickness of this region is controlled by fluid dynamics; faster water leads to increased shear stress and a thinner DBL²⁰⁻²³.

As described in the results section, for steady flow over a flat surface the DBL thickness can be estimated from the VBL thickness or friction velocity using the Schmidt number:

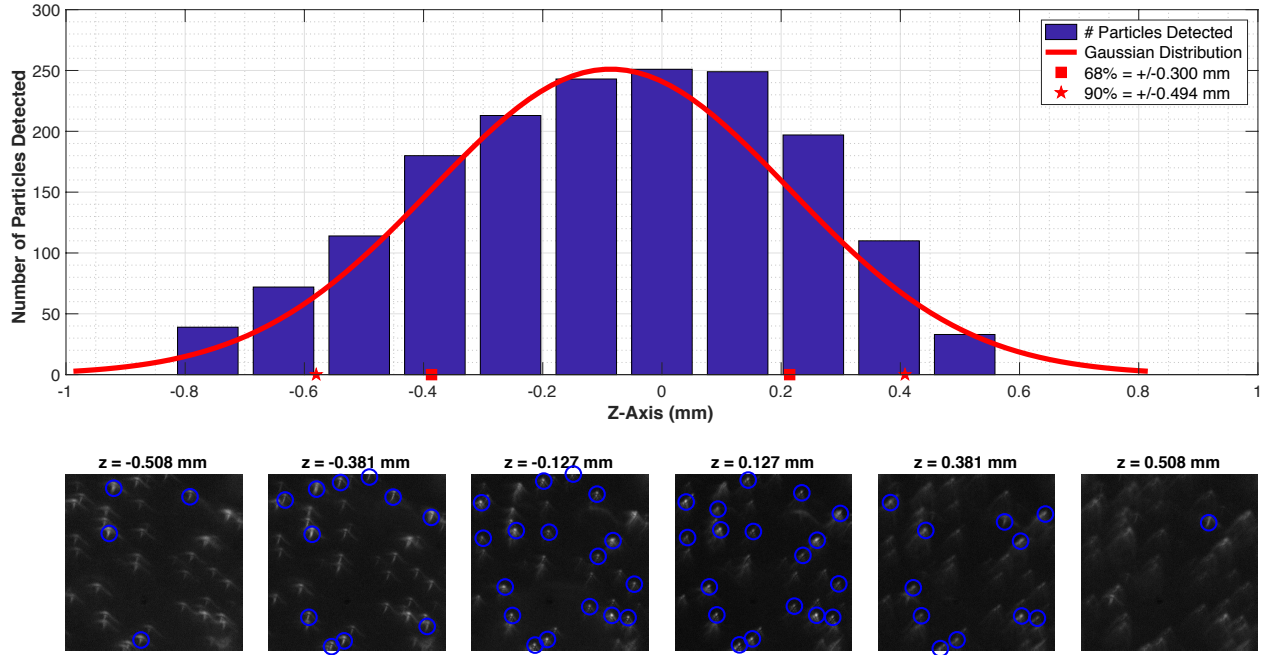
$\delta_{DBL} \approx \delta_{VBL} (Sc^{-\frac{1}{3}}) \approx 10 \frac{\nu}{u_*} (Sc^{-\frac{1}{3}})$.²⁰⁻²² For typical dissolved gases and nutrients in water at ambient temperatures $Sc \approx 500$ to $1,000$; and it is therefore expected that the DBL thickness is approximately one tenth the VBL thickness ($\delta_{DBL} \sim \delta_{VBL} \frac{1}{10}$).^{4,20-22} However caution must be taken when applying these idealized scaling relationships to natural environments (which have non-stationary flows and rough surfaces. Careful lab flume tests measuring chemical gradients and fluid velocities above marine sediments found the scaling relationship between DBL thickness and friction velocity to be valid, although the leading coefficient varied^{21,22}. The relationship between shear stress and mass transfer has also been investigated over complex coral surfaces using numerical simulations; model results showed that local shear stress is a proxy for local mass flux, and a second order polynomial was used to fit this relationship²³. Additionally, studies considering the DBL under for non-stationary flows have shown it may be important to consider relevant time scales of diffusion and velocity fluctuations^{14,18}

Here a simple scaling relationship ($\delta_{DBL} \sim 10 \frac{\nu}{u_*} Sc^{-\frac{1}{3}}$, with $Sc=1,000$) is used to determine an approximate, order-of-magnitude DBL thickness. This is only an order-of-magnitude approximation as mass transfer over rough surfaces such as corals is complex and the μ PTV system does not resolve out-of-plane flows so only a lower bound on friction velocity is calculated.

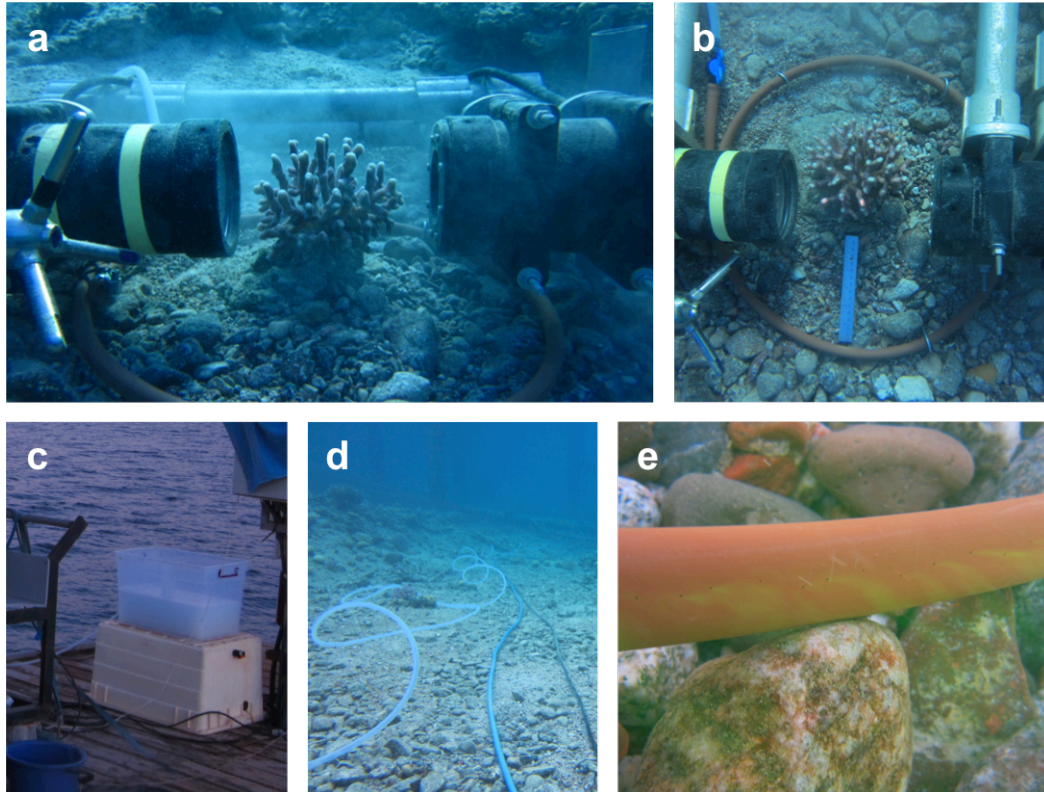
3.6 Supplementary



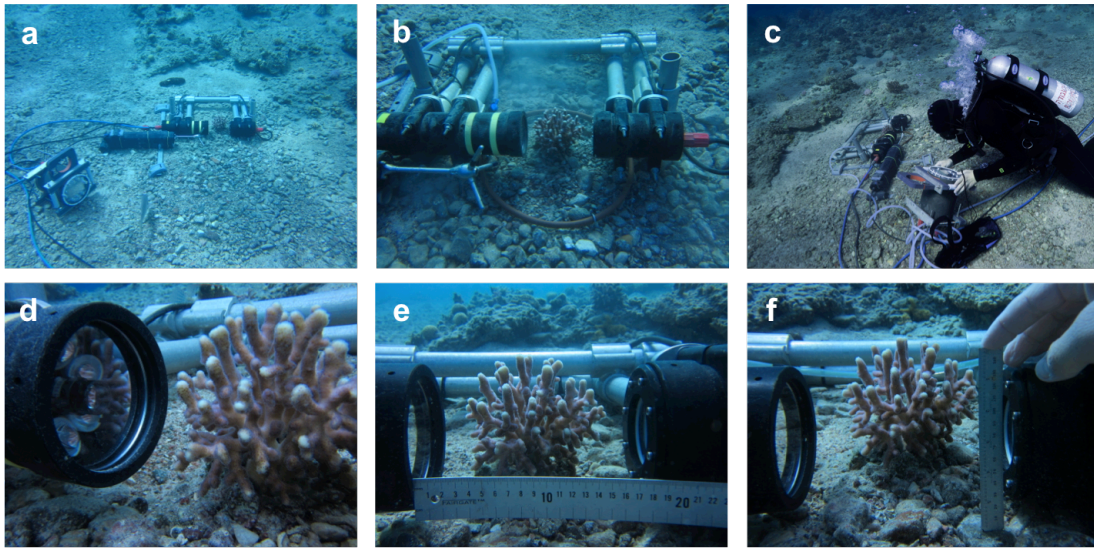
Supplementary Figure 3.1 | Schematic of μ PTV optics and timing diagram. (a) Optical schematic of the μ PTV system (not to scale), showing the dark-field illuminator on the right and the underwater microscopic imaging system on the left. The dark field illumination path is shown, and it can be seen that only light forward scattered in the imaging plane is captured by the microscopic objective lens. The spacing of the housings is also labeled. (b) Timing diagram showing the illumination and camera exposure timing. The camera was equipped with an inter-line CCD architecture which allowed consecutive frame acquisition with very short time intervals. A frame straddling image acquisition approach is used, in which the camera is exposed for two long intervals with a very short inter-frame time. The illumination is flashed at the end of frame one and beginning of frame two, and these illumination flashes determine the time points at which the images are acquired.



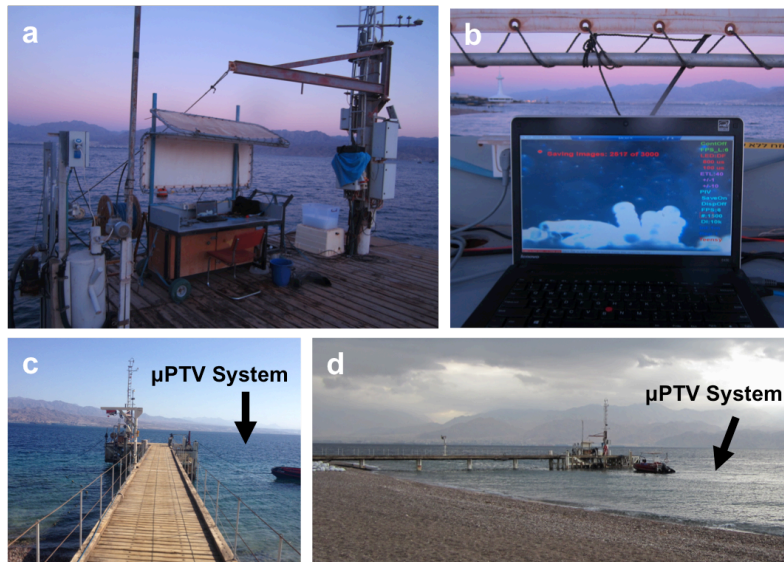
Supplementary Figure 3.2 | Measurement plane width calibration. The thickness of the μ PTV measurement plane (the volume over which the μ PTV system detects particles), was calibrated by imaging a microscopic slide with many particles. Images were acquired while the particle slide was translated in the z-axis using a micromanipulator. The particle detection algorithm was then applied to each image and all detected particles were counted. The top graph shows the number of particles detected as a function of position in the z-axis. A Gaussian curve was fit to this distribution to estimate the thickness of the plane over which particles were are detected. The bottom graph shows a sub-sample of the image field of view (500x500 pixels) at several different z-positions, with detected particles circled in blue. All imaging was conducted in the lab flume



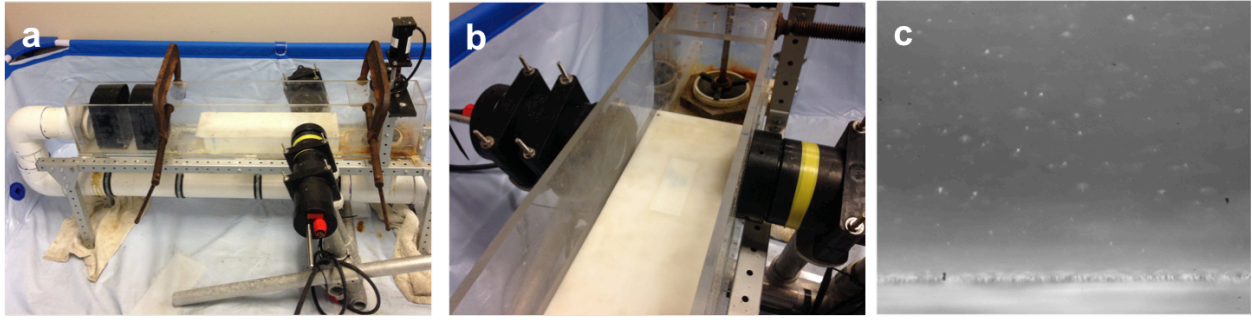
Supplementary Figure 3.3 | Active particle seeding setup. (a,b) Active *in situ* particle seeding around a coral colony. A mixture containing seawater and 10 μm hollow glass spheres is being slowly emitted from rubber tubing that is looped around the coral. The ruler shown in **b** for scale is 12.7 cm (5 in) long. (c) Seawater-particle mixture on the IUI pier. Seawater was collected directly from ocean and mixed with tracer particles in the plastic tub, then channeled to the coral colony using plastic hosing. (d) Left, hosing channeling the seawater-particle mixture to the μPTV system, right blue electronic cable for remote μPTV instrument operation. (e) Seawater-particle mixture slowly being emitted into surrounding water through small needle holes in the rubber tubing. Holes were pointed away from center of the tubing and coral. In **e** water was colored with fluorescein dye to make it easily visible for testing purposes, under normal operations this fluorescein was not added.



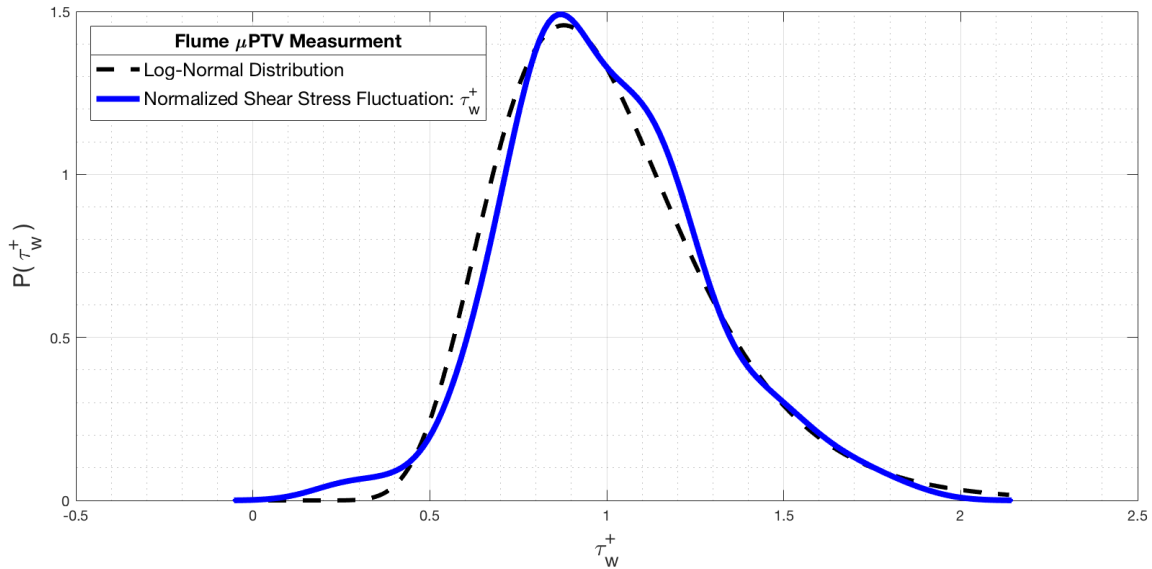
Supplementary Figure 3.4 | Underwater μ PTV setup. (a-f) Images of the μ PTV system deployed *in situ* around a small coral colony. (a) The underwater computer, microscopic imaging housing, dark-field illumination housing, and frame can all be seen here. (b) A close up image of the μ PTV setup. The three pronged device in front of the imaging housing is an ADV measuring velocities in the water near the coral, and the circular hosing around the coral is being used for particle seeding. (c) Underwater diver operation of instrument. (d) Imaging optics pointed at the coral. (e) Ruler showing distance between housing (labeled markings are in centimeters). (f) Ruler for scale is 12.7cm (5 in) long.



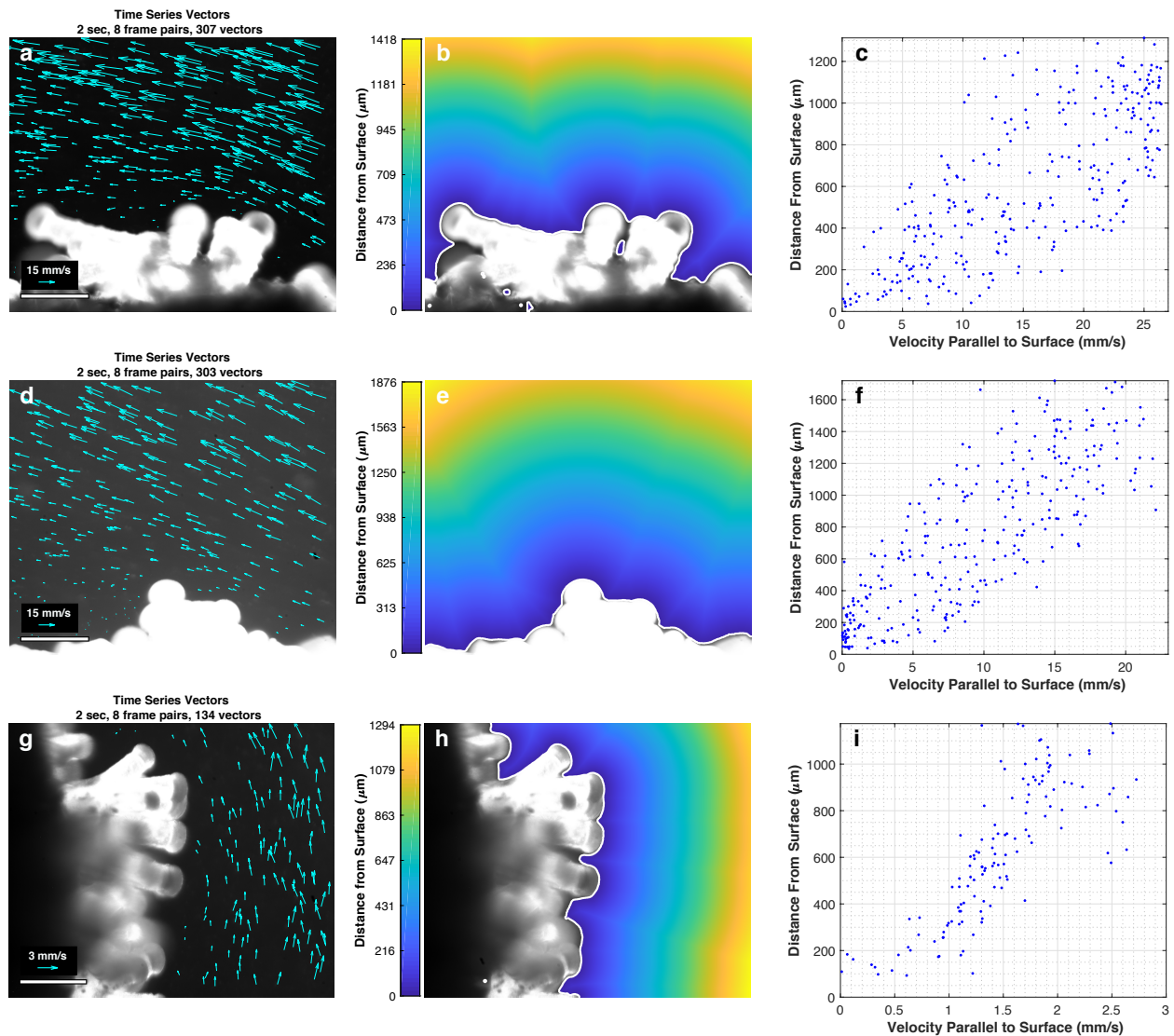
Supplementary Figure 3.5 | Field site & remote μ PTV operations. (a) Remote μ PTV operations setup at the end of the IUI pier. (b) Computer providing live image feed and control of underwater μ PTV instrument in real time. (c,d) Location of instrument underwater near the end of the IUI pier.



Supplementary Figure 3.6 | Lab flume setup. (a,b) Lab flume based on Vogel design (channel approximately 122 cm long x 15 cm wide x 20 cm deep). μ PTV system placed around flume observing flow over a flat, knife-edged plate machined from Delrin. (c) Example of particle field image acquired by μ PTV system above the flat plate in the flume.



Supplementary Figure 3.7 | PDF of normalized shear stress fluctuations measured in the lab flume. The μ PTV system was used to measure instantaneous fluctuations in shear stress at the surface of a flat plate in the lab flume. 200 μ PTV frame pairs were captured at a rate of 4 frame pairs per second while the flume was operating at flow speed of approximately 90 mm/s. The instantaneous shear stress was then calculated for each frame pair using a linear fit to the near surface velocities. Approximately 16% of these measurements were thrown out due to low particle concentrations in the near surface region or a poor linear fit likely caused by spurious vectors. A PDF of normalized instantaneous shear-stress ($\frac{\tau_w}{\tau_w}$) was calculated and is shown above. The PDF closely follows a log-normal distribution, matching previous experimental studies. The normalized intensity of shear stress fluctuations was also calculated: $\tau_{w,rms}^+ = \frac{(\tau_w - \overline{\tau_w})_{rms}}{\overline{\tau_w}} \approx 0.28$ (i.e. the instantaneous shear stress typically fluctuates approximately 28 % from the mean). This value matches previously reported measurements in the literature varying from 0.28 to 0.43. The measurement of shear stress fluctuation intensity is likely slightly reduced here as a result of spatial averaging due to the μ PTV system measuring a finite volume.



Supplementary Figure 3.8 | *In situ* μ PTV vector fields and transformation to wall coordinates. *In situ* vectors collected over 8 frame pairs (recorded over 2 seconds). The three rows here correspond to the rows in Fig. 3.4 as well as Supplementary Videos 3.1-3.3 (**a, d g**) All vectors measured from 8 μ PTV frame pairs around the *in situ* coral polyp, scale bar 500 μ m. (**b,e,h**) Closest distance from each point in the image to the coral polyp surface. (**c, f, i**) Vectors transformed into a one-dimensional profile. A distance from the coral's surface is determined for each vector. Additionally the component of each vector parallel to the surface is calculated. This provides a velocity profile parallel to the coral's surface as a function of distance from the surface.

Supplementary Video 3.1 | *In situ* μ PTV imaging and particle tracking 3.1. Video corresponds to data sets used to make Fig. 3.4 a,b and Supplementary Fig. 3.8 a-c. Video shows *in situ* μ PTV measurements of fluid velocities around coral polyps in a natural reef environment. Images were recorded in frame pairs: orange circles identify particles in frame #1, blue circles identify particles in frame #2. Arrows show velocity vectors determined by particle displacements between frames. Arrow lengths are in proportion to their speed and a reference vector arrow is displayed in the bottom left of the video. Video top left shows a time stamp and bottom left shows a 500 μ m scale bar. Images were recorded at 4 frame pairs per second, and each video shows 8 μ PTV frame pairs (each data set recorded over a 2 second time period). 2 seconds of particle tracking (8 vector fields). Images in videos are strongly contrast enhanced in order to increase particle visibility for display purposes.

Supplementary Video 3.2 | *In situ* μ PTV imaging and particle tracking 3.2. Video corresponds to data sets used to make Fig. 3.4 c,d and Supplementary Fig. 3.8 d-f. Video shows *in situ* μ PTV measurements of fluid velocities around coral polyps in a natural reef environment. Images were recorded in frame pairs: orange circles identify particles in frame #1, blue circles identify particles in frame #2. Arrows show velocity vectors determined by particle displacements between frames. Arrow lengths are in proportion to their speed and a reference vector arrow is displayed in the bottom left of the video. Video top left shows a time stamp and bottom left shows a 500 μ m scale bar. Images were recorded at 4 frame pairs per second, and each video shows 8 μ PTV frame pairs (each data set recorded over a 2 second time period). 2 seconds of particle tracking (8 vector fields). Images in videos are strongly contrast enhanced in order to increase particle visibility for display purposes.

Supplementary Video 3.3 | *In situ* μ PTV imaging and particle tracking 3.3. Video corresponds to data sets used to make Fig. 3.4 e,f and Supplementary Fig. 3.8 g-i. Video shows *in situ* μ PTV measurements of fluid velocities around coral polyps in a natural reef environment. Images were recorded in frame pairs: orange circles identify particles in frame #1, blue circles identify particles in frame #2. Arrows show velocity vectors determined by particle displacements between frames. Arrow lengths are in proportion to their speed and a reference vector arrow is displayed in the bottom left of the video. Video top left shows a time stamp and bottom left shows a 500 μ m scale bar. Images were recorded at 4 frame pairs per second, and each video shows 8 μ PTV frame pairs (each data set recorded over a 2 second time period). 2 seconds of particle tracking (8 vector fields). Images in videos are strongly contrast enhanced in order to increase particle visibility for display purposes.

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Chapter 4

In situ micro-scale fluid dynamics at the coral-ocean interface

4.1 Abstract

Corals need to obtain and eliminate solutes such as oxygen and nutrients in order to conduct basic metabolic functions. Small-scale water motions at the coral's surface control rates of chemical flux to and from the surrounding environment, and are significant to organism health. However fluid motions on the reef are complex and difficult to fully replicate in the lab. Here a novel underwater micro-particle tracking velocimetry (μ PTV) system is used to measure two-dimensional fluid velocity fields at micro-scales around individual coral polyps *in situ*. Time series analysis and ensemble averaging show significant periodic fluctuations in velocities and shear stress at the coral's surface. Comparisons with nearby flow measurements indicate that near-surface velocity fluctuations are being driven by oscillating flows on the reef caused by small amplitude surface waves. Measured peaks in shear stress are expected to enhance mass exchange between the coral and surrounding water. For the observations performed here, we find that periodic velocity peaks likely have a more significant influence on mass transfer than time-averaged velocities.

4.2 Introduction

Reef building corals are fundamentally influenced by the water motions surrounding them^{1,2}. In order to conduct diverse metabolic functions needed to build reef habitats, the coral organism must continuously exchange materials with the surrounding seawater³. Exchanges include transfer of dissolved oxygen, carbon dioxide, nutrients, calcium carbonate, and elimination of toxic reactive oxygen species. Fluid motions at the coral surface-water interface control mass flux rates of these materials to and from the coral, as a result the flow environment has strong impacts on basic physiological processes^{1,3-5}.

Water motions are especially significant at scales of millimeters or less from the coral's surface. Here a viscous boundary layer (VBL) forms^{1,3,6} in which viscous forces dominate momentum transfer. Average fluid velocities in this region increase linearly from the surface and this velocity gradient can be used as a direct measure of wall shear stress⁷⁻¹⁰. Due to low mixing, at scales of hundreds of micrometers from the surface a chemical diffusive boundary layer (DBL) forms, in which the transport of dissolved gases is dominated by molecular diffusion^{5,11,12}. The DBL impedes mass flux and may act as a rate-limiting factor for basic metabolic functions; this has been shown to influence processes including photosynthesis and respiration^{4,11,12}, calcification^{13,14}, and coral-algae competition^{15,16}. Significantly, fluid dynamics at the coral's surface control the thickness of this DBL, and thus mass flux rates^{3,9,17,18}.

A history of experiments on corals have shown that greater fluid motion in the surrounding water increases mass transfer and is positively correlated with many aspects of coral health^{5,19-24}, including their ability to deal with climate change stressors of ocean warming²⁵ and acidification²⁶. Investigations of water motions over and through coral colonies have been conducted with a variety of techniques including: hot wire anemometry²⁷, particle imaging

velocimetry^{4,28,29}, laser Doppler velocimetry³⁰, acoustic Doppler velocimetry²⁹, magnetic resonance velocimetry³¹, and direct numerical simulations¹⁷. Bulk relationships between large-scale flows and mass transfer rates over corals have also been studied using engineering parameterizations and gypsum dissolution experiments³²⁻³⁷. These investigations have shown that waves and bottom roughness enhance mass exchange on coral reefs in the ocean, that flow through the coral colony structure is complex, and that local shear stress acts as a proxy for mass transfer. Additionally, in the lab, micro-particle tracking velocimetry (μ PTV) has been used to observe micro-scale fluid motions at the surfaces of coral fragments held in small milli-fluidic chambers, revealing coral cilia driven mixing³⁸. However no field observational techniques have provided sufficient spatial resolution to observe fluid motions and shear stress within the critical VBL at the coral's surface *in situ*, nor to observe flow around individual coral polyps in the ocean.

Local water motions on coral reefs are a complex combination of currents, waves, and eddies². These in turn flow over corals, which have intricate structure at the scale of the colony, branch, and polyp³⁹. Interactions between natural reef flows and coral structure ultimately control the near-surface fluid motions and as a result mass transfer to and from corals. A need thus exists for small-scale measurements of fluid velocities at the coral-water interface in the ocean.

Here a new underwater μ PTV system is used to collect novel measurements of fluid velocities at sub-millimeter scales directly at the surface of a coral in a natural reef environment. Using these field observations we examine water motions at the coral polyp scale and investigate: 1) the velocity profile at the coral's surface, 2) temporal variability in the micro-scale flow environment, 3) the influence of surrounding water motions on the near-surface

velocities, and 4) the impacts of morphology and polyp structure on near surface flows. Results show significant periodic oscillations in shear stress at the coral's surface driven by small amplitude surface waves on the reef.

4.3 Results

4.3.1 Underwater μ PTV

A new underwater μ PTV system was used to collect quantitative measurements of two-dimensional velocity fields surrounding individual coral polyps *in situ*. Field work was conducted in the Gulf of Eilat, northern Red Sea, offshore of the Interuniversity Institute for Marine Sciences (IUI). This location typically experiences currents of approximately 10 cm/s per second or less, and small amplitude surface waves. Here the μ PTV system was deployed by scientific divers on sandy seafloor at approximately 5 m depth and positioned around a small naturally occurring coral colony of *Pocillopora spp.* (Fig. 1).

The underwater μ PTV instrument captured microscopic images at the coral-water interface. Fields of two component fluid velocity vectors were then determined by tracking tracer particles in the water between sequential microscopic images (details in Methods) (images were recorded at 4 frame pairs per second). Fluid velocities and pressure were also measured nearby using an acoustic Doppler velocimeter (ADV) placed approximately 15 cm away from the coral. The analysis presented here focuses on two time series μ PTV measurements of fluid velocities recorded around the same coral polyp approximately 2.5 hours apart. (Data Set #4.1: polyp contracted – 50 sec, 200 frame pairs; Data Set #4.2: polyp expanded – 24 sec, 96 frame pairs; vector field time series are shown in Supplementary Video 4.1, 4.2)

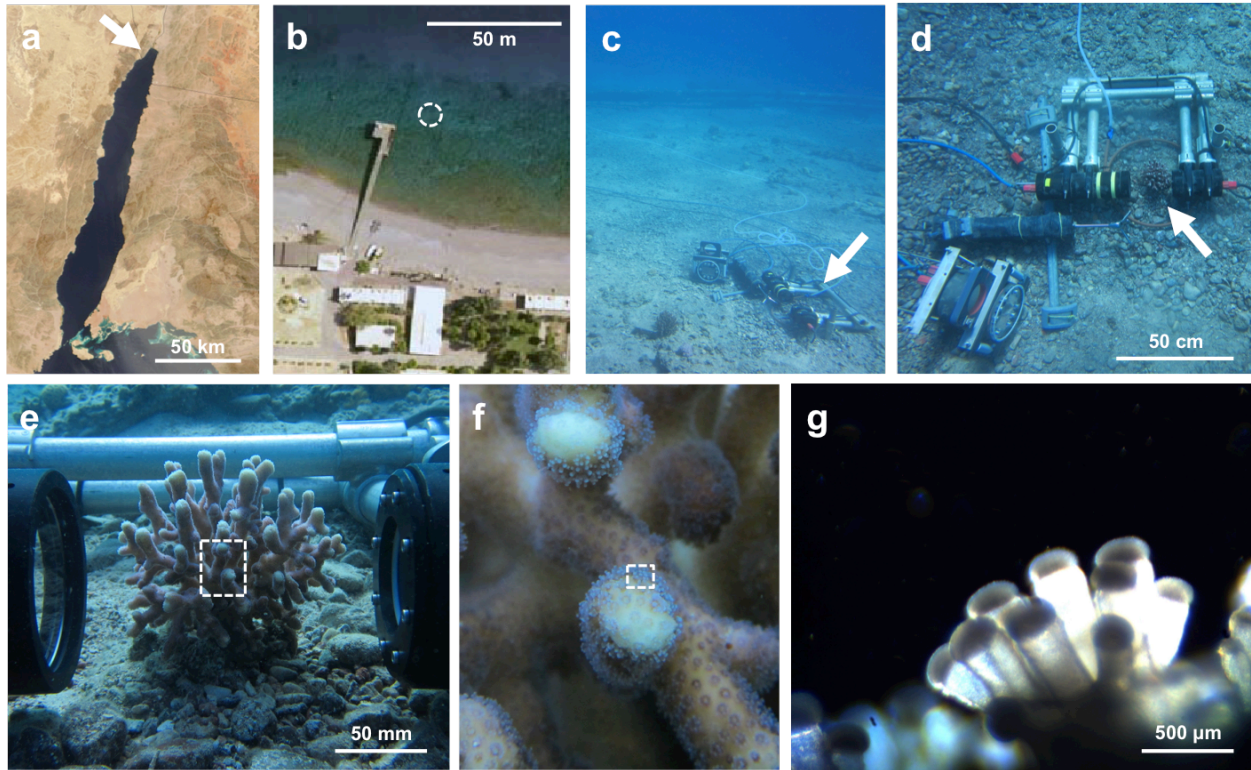


Figure 4.1 | Field site & μ PTV system. (a) Satellite view of the in the Gulf of Eilat (approximately 160 x 24 km) which extends off of the Red Sea. Arrow points to Eilat, where *in situ* deployments were conducted. (Image from NASA) (b) Aerial view of the field site with circle showing approximate location of underwater μ PTV system. Underwater fieldwork was conducted approximately 30 m offshore of the Interuniversity Institute for Marine Sciences. (Image reproduce from Ben-Shaprut & Goodman-Tchernov⁴⁰ with permission) (c-d) The μ PTV system was deployed on a sloping, sandy bottom with sparse coral cover at a depth of approximately 5 m. The setup consisted of an underwater microscopic imaging system, dark field illumination, underwater computer, and active particle seeding. Additionally, an ADV was placed approximately 15 cm from the imaging volume. Arrow points to coral subject being imaged. (e) The μ PTV system was positioned around a small naturally occurring colony of *Pocillopora spp.*, with approximately 95 mm of space between both optical housings and the imaged volume. Box corresponds approximately to area shown in f and shows general location on colony within which the μ PTV system is imaging. (f) Macro-image showing coral structure at the scale of the branch and polyp. Box shows approximate area imaged by microscope (box does not correspond to microscope's specific field-of-view). (g) *In situ* microscopic image observing coral polyps and surrounding water. The system imaged a field-of-view of approximately 2.5 x 2.1 mm.

4.3.2 Time averaged velocity fields

The micro-flow environment at the coral's surface was first examined using a time-averaged approach. For each time series all μ PTV vectors were combined. An average velocity magnitude at each two-dimensional position in the field-of-view was then calculated based on a weighted average of the magnitude of all vectors within a 400 μm radius (Fig. 4.2 a,c; details in Methods). An average velocity profile was also calculated based on velocity magnitude parallel to the coral's surface as a function of distance from the surface, and a linear fit was applied to velocities 0 to 600 μm from the coral (Fig. 4.2 b,d; details in Methods).

Two-dimensional fields showing time-averaged fluid velocity magnitude reveal a strong gradient in flow speed at the coral's surface (Fig. 4.2 a,c). The time-averaged velocity profile shows an approximately linear increase in speed with distance from the coral in the near surface region (Fig 4.2 b,d). This matches predicted theory and indicates that fluid motions are being observed within a VBL at the coral's surface. The velocity gradient in this linear region ($\frac{d\bar{u}}{dn}$, where $\bar{u}$ is the average velocity parallel to the surface, and n is distance from the surface) can be used as a direct measure of the wall shear stress ($\tau_w = \mu \frac{\partial \bar{u}}{\partial n} \approx \mu \frac{d\bar{u}}{dn}$, where μ is dynamic viscosity) and the friction velocity ($u_* = (\tau_w / \rho)^{1/2}$, where ρ is density)^{7-10,41-43}. Additionally, applying scaling laws and assuming steady flow, the friction velocity can be used to estimate an approximate order-of-magnitude DBL thickness ($\delta_{\text{DBL}} \sim 10 \frac{\nu}{u_*} \text{Sc}^{-\frac{1}{3}}$, details in Methods)^{9,18,44}. However it can also be seen that, while the time averaged velocity profile is approximately linear, the instantaneous velocity vectors in the time series show significant variability (Fig. 4.2 b,d). This indicates that the flow is likely non-stationary, and it is therefore important to consider temporal dynamics when estimating DBL thickness and rates of chemical flux.

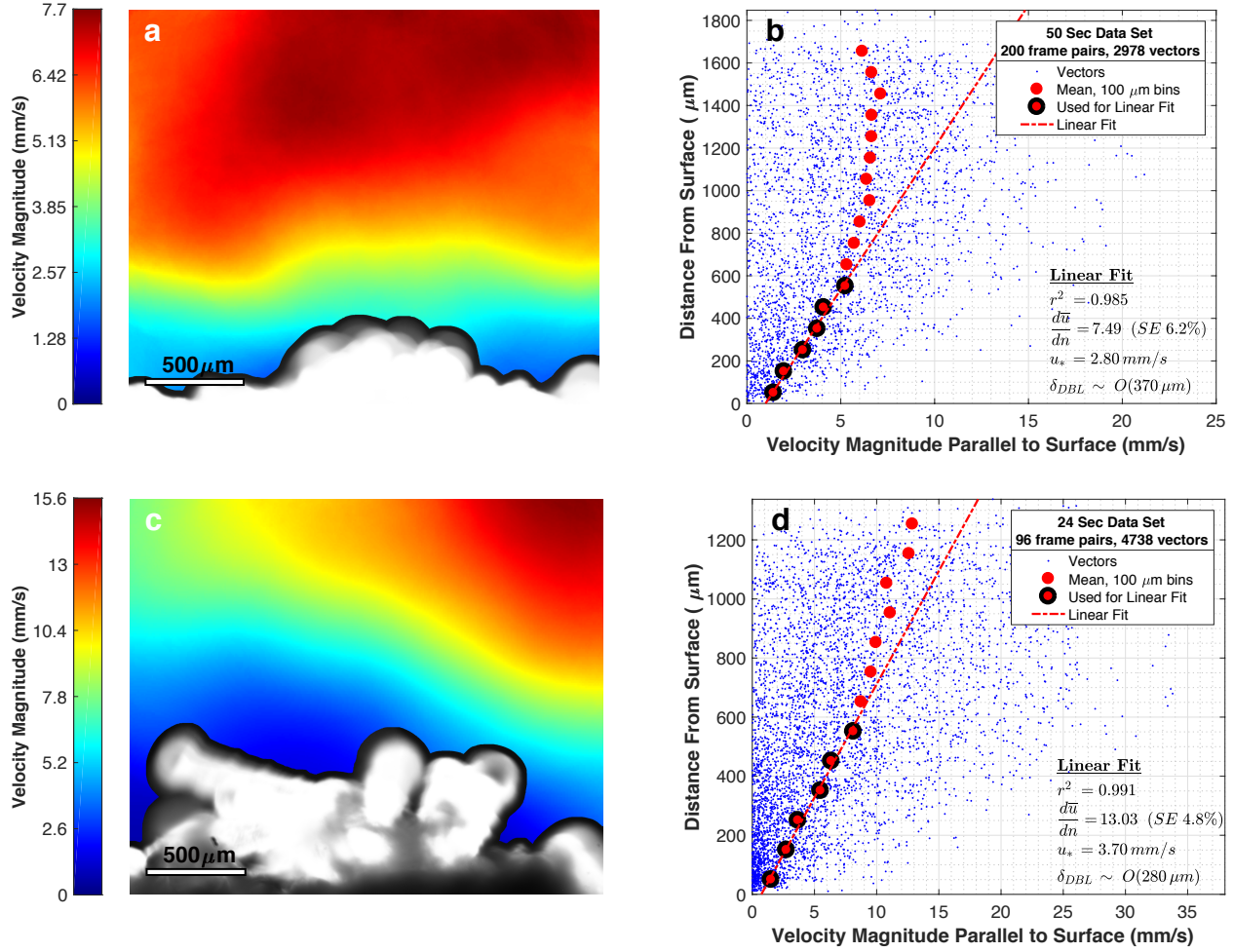


Figure 4.2 | Time-averaged velocity field. (a,c) Two-dimensional time-averaged velocity magnitude at the coral's surface measured *in situ*. Velocity at each point is a weighted average of the absolute magnitude of all vectors in the time series within a 400 μm radius. Instrument field of view is approximately 2.5 x 2.1 mm. (b,d) Magnitude of velocity parallel to the coral's surface ($|\bar{u}|$) as a function of distance from the coral's surface (n). Vectors binned every 100 μm and averaged. A linear fit is then applied to the first 6 bins in order to determine an average velocity gradient. The figure text reports the linear fit coefficient of determination (r^2), the gradient ($\frac{d\bar{u}}{dn}$), and the standard error of the gradient slope fit (SE). The velocity gradient is used to calculate friction velocity (u_*) and provide and an approximation of DBL thickness (δ_{DBL}). The legends report the number of image pairs and vectors collected over the time series and used to create the figures. Top row (a,b) correspond to Data Set #4.1 and bottom row (c,d) correspond to Data Set #4.2. Scale bar 500 μm.

4.3.3 Temporal fluctuations in near-surface flows

In order to examine the temporal variability in the near-surface flow, a mean horizontal velocity and linear velocity gradient were calculated for each individual vector field (Fig. 4.3a,c, 4.4a,c; details in Methods). Spectral analysis was then performed on the time series in order to investigate the frequency components of these signals (Fig. 4.3b,d, 4.4b,d). Additionally, vector field videos show qualitative changes in flow with time (Supplementary Video 4.1, 4.2).

In situ water motions at the coral's surface are clearly non-stationary. Both the mean horizontal fluid velocity and the velocity gradient exhibit strong fluctuations occurring at distinct frequencies. Two different flow regimes were observed. In Data Set #4.1 near surface water motions experienced periodic reversals in direction every few seconds (Fig. 4.3). While in Data Set #4.2 the flow was unidirectional, but still had strong periodic fluctuations in strength (Fig. 4.4).

Fourier analysis of the unidirectional flow shows that the horizontal velocity and the velocity gradient have peak fluctuations at a matching frequency – one peak in shear stress every wave cycle (Fig 4.4b,d). While for the reversing flow, the velocity gradient has a peak fluctuation at twice the frequency of the horizontal velocity – two peaks in shear stress every wave cycle (Fig. 4.3b,d). In both cases the instantaneous velocity gradient was positively correlated with the mean horizontal fluid velocity magnitude (Data Set #4.1: $r^2 = 0.62$; Data Set #4.2: $r^2 = 0.76$; Supplementary Fig. 4.1). These time series μ PTV results indicate that periodic fluctuations in velocity at the coral's surface are driving changes in shear stress; with faster water causing greater shear and as a result expected decreases in DBL thickness.

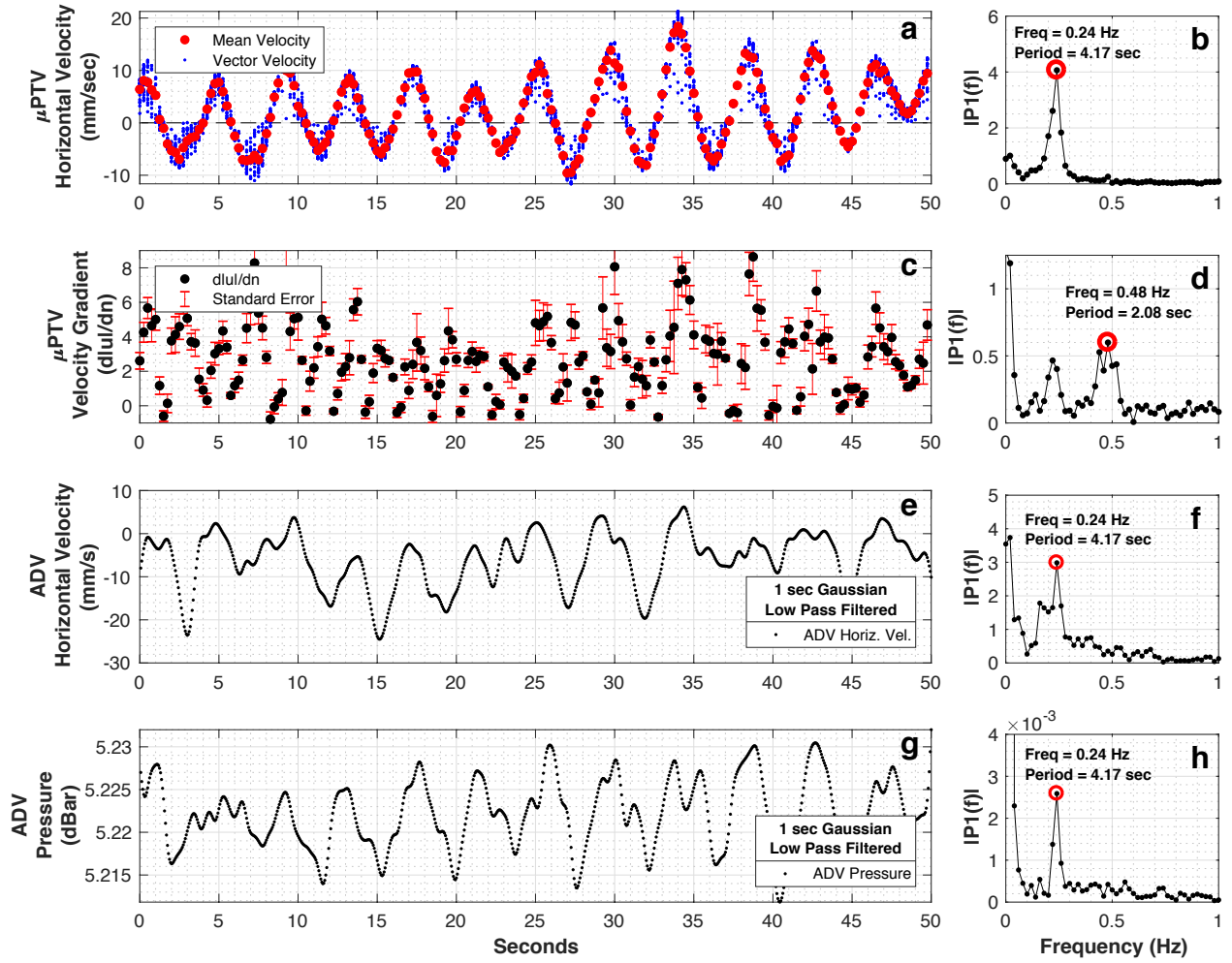


Figure 4.3 | μ PTV & ADV temporal fluctuations, Data Set #4.1. Left column shows time series data and right column shows corresponding Fourier transforms. (a,b) Mean horizontal velocity for each μ PTV vector field. (c,d) Linear velocity gradient fit to each μ PTV vector field. (e,f) ADV horizontal velocity, in the same direction as the μ PTV horizontal measurement plane. (g,h) ADV pressure. μ PTV data was collected at 4 Hz; ADV data was collected at 16 Hz and convolved with a 1 sec Gaussian filter to reduce noise. Raw ADV data shown in Supplementary Fig. 4.2.

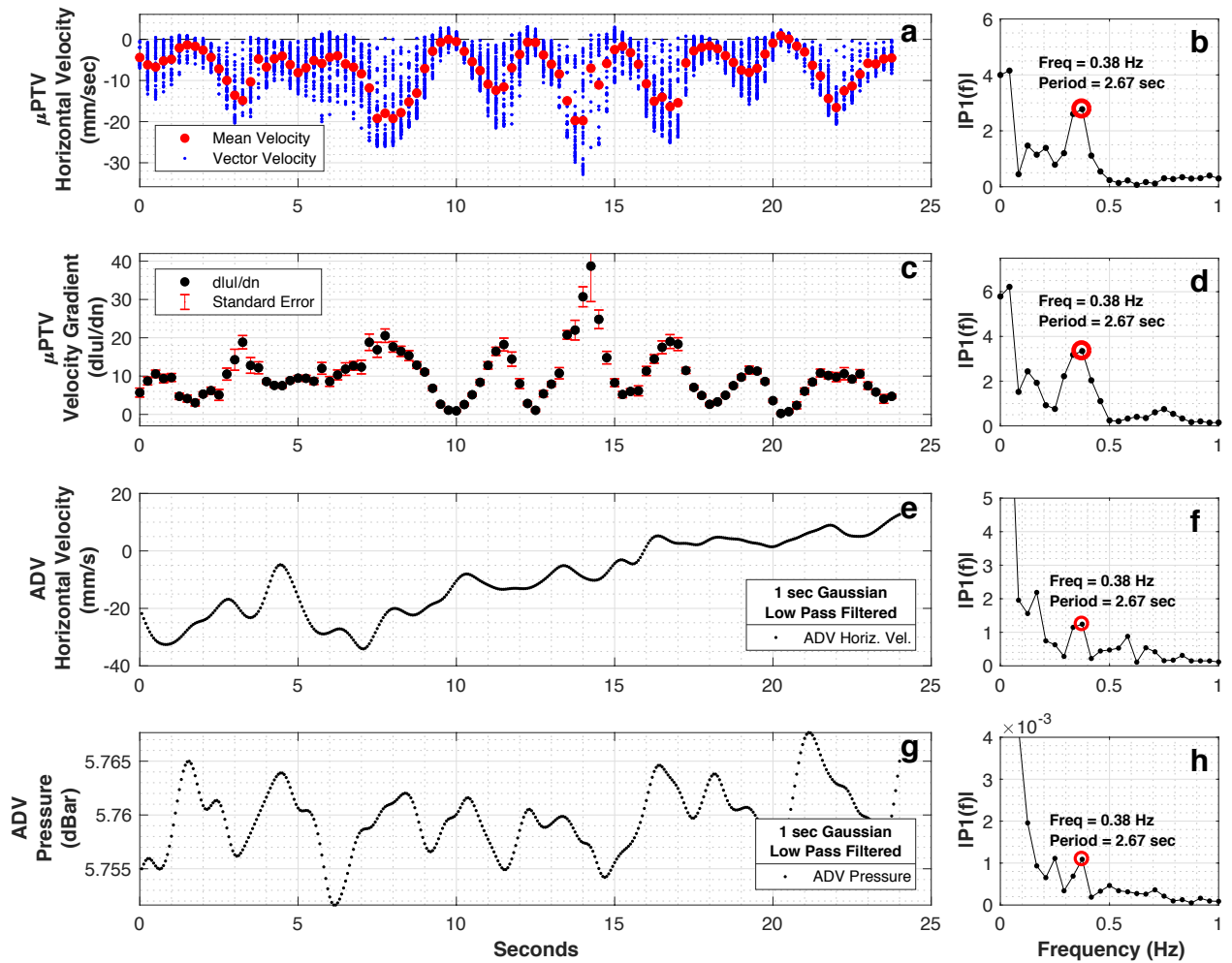


Figure 4.4 | μ PTV & ADV temporal fluctuations, Data Set #4.2. Left column shows time series data and right column shows corresponding Fourier transforms. (a,b) Mean horizontal velocity for each μ PTV vector field. (c,d) Linear velocity gradient fit to each μ PTV vector field. (e,f) ADV horizontal velocity, in the same direction as the μ PTV horizontal measurement plane. (g,h) ADV pressure. μ PTV data was collected at 4 Hz; ADV data was collected at 16 Hz and convolved with a 1 sec Gaussian filter to reduce noise. Raw ADV data shown in Supplementary Fig. 4.3.

4.3.4 Outer flow, waves & inner flow

Concurrent μ PTV and ADV measurements provide a means to compare small-scale water motions at the coral's surface with flows at larger-scales in the surrounding water. The outer flow, as measured by the ADV, showed velocities of several centimeters per second with strong periodic oscillations (Fig. 4.3e-f, 4.4e-f; raw ADV data Supplementary Fig. 4.2, 4.3). Peak oscillating frequencies measured by the ADV match those measured by the μ PTV system, indicating that large-scale water motions and oscillations in the outer flow are driving oscillations in velocities at the coral-water interface. The ADV pressure sensor additionally shows these same peak frequencies (Fig. 4.3g-h, 4.4g-h). The pressure data provides evidence that the oscillations in the outer flow as well as the small-scale flow at the coral's surface are specifically being driven by ocean surface waves.

The wavelength of the surface waves modulating the system can be calculated from the wave frequency and coral depth using linear wave theory. Additionally, wave height can be estimated from linear wave theory based on the pressure fluctuations (Supplementary Fig. 4.4, 4.5; details in Methods)^{45,46}. (Data Set #4.1: wave period ≈ 4.3 s, wavelength ≈ 24 m, significant wave height ≈ 4.1 cm. Data Set #4.2: wave period ≈ 2.8 s, wavelength ≈ 11 m, significant wave height ≈ 5.0 cm.) Measurements recorded here are typical for reefs in Eilat, which normally have flow speeds of 5-10 cm/s^{47,48} and small amplitude waves. Oscillation periods observed here (2.5-4.5 sec) also match previous observations of wave periods in Eilat^{28,49}.

In summary, Fig 4.3 and 4.4 indicate that relatively small amplitude surface waves are producing fluctuations in flow speeds near the seafloor at 5m depth. These oscillations in flow are in turn driving significant fluctuations in shear stress as well as flow reversals within the coral VBL.

4.3.5 Wave phase ensemble average

The impact of a typical wave on the μ PTV velocity field was then examined using an ensemble averaging approach. Here we combined vectors recorded at the same wave phase over several wave cycles^{30,50-52}. This allowed us to build up a dense vector field for each phase and to investigate the impact of a wave on the VBL averaged over several iterations. First an instantaneous wave phase was calculated for each μ PTV image pair by applying a Hilbert transform to the mean horizontal velocity time series (Supplementary Fig. 4.6, 4.7)^{50,53,54}. Vector fields were then divided into 8 groups based on their instantaneous phase, and an average velocity field was calculated for each phase (Fig. 4.5, 4.6, Supplementary Video 4.3, 4.4).

Vectors in each phase were also used to calculate an ensemble mean horizontal velocity, velocity profile, and shear stress as a function of wave phase (Fig. 4.7, 4.8). The mean horizontal velocity as a function of wave phase approximates a sinusoidal function (Fig. 4.7b, 4.8b). As mean velocities increase the corresponding average velocity profile becomes steeper indicating greater shear stress and a thinner, compressed VBL (Fig. 4.7c, 4.8c). A velocity gradient and corresponding shear stress were calculated for each wave phase profile based on a linear fit to the first six average velocities (i.e. 0 to 600 μ m from the coral surface) (Fig. 4.7d, 4.8d). Additionally friction velocities calculated based on the phase maximum, phase minimum, and time averaged shear stresses are reported.

Ensemble averaging shows peak velocities driving higher maximum shear stress and friction velocity than time averaged values (Fig. 4.6, Fig. 4.7). For Data Set #4.1 using a time averaged approach: $u_{*avg} = 2.80$ mm/s, and for ensemble averaging: $u_{*max} = 3.58$ mm/s. For Data Set #4.2: $u_{*avg} = 3.70$ mm/s, and $u_{*max} = 5.46$ mm/s. Here peak flow speeds are thus causing increases in friction velocity by approximately 25% to 50% over time-averaged values.

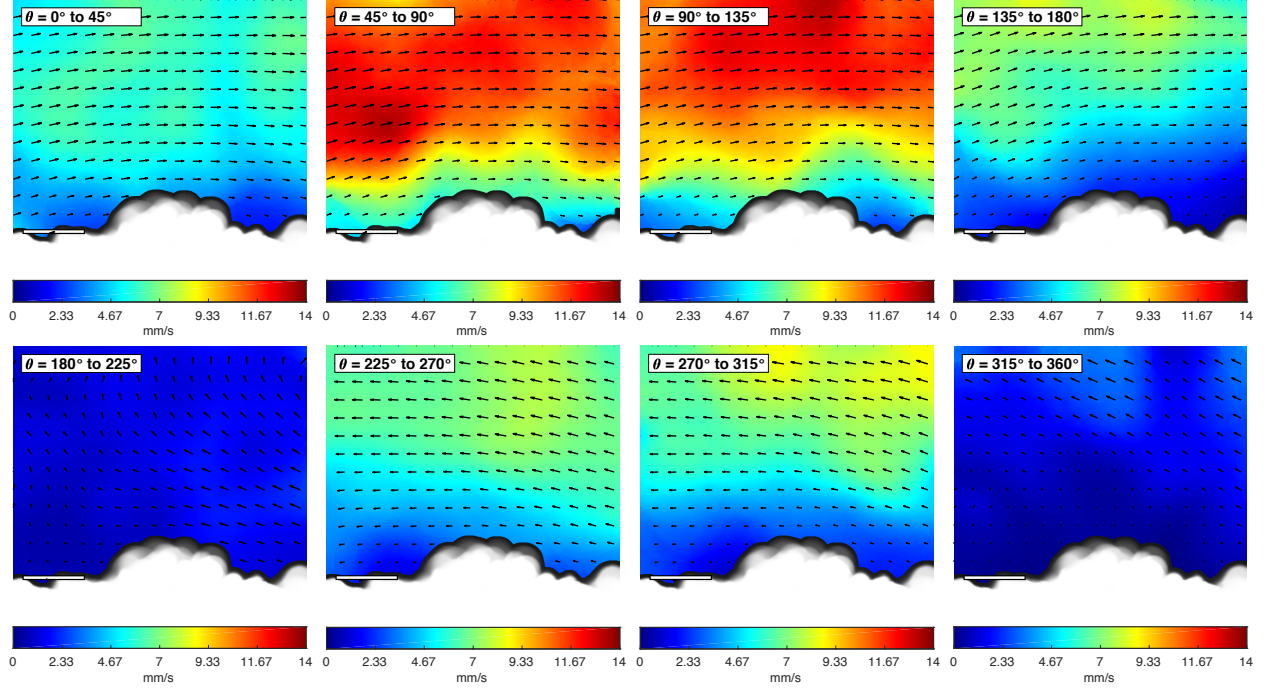


Figure 4.5 | Ensemble average velocity fields as a function of wave phase, Data Set #4.1. Two-dimensional velocity field calculated for each wave phase group using an ensemble average approach. Legend reports range of wave phases used in each ensemble.

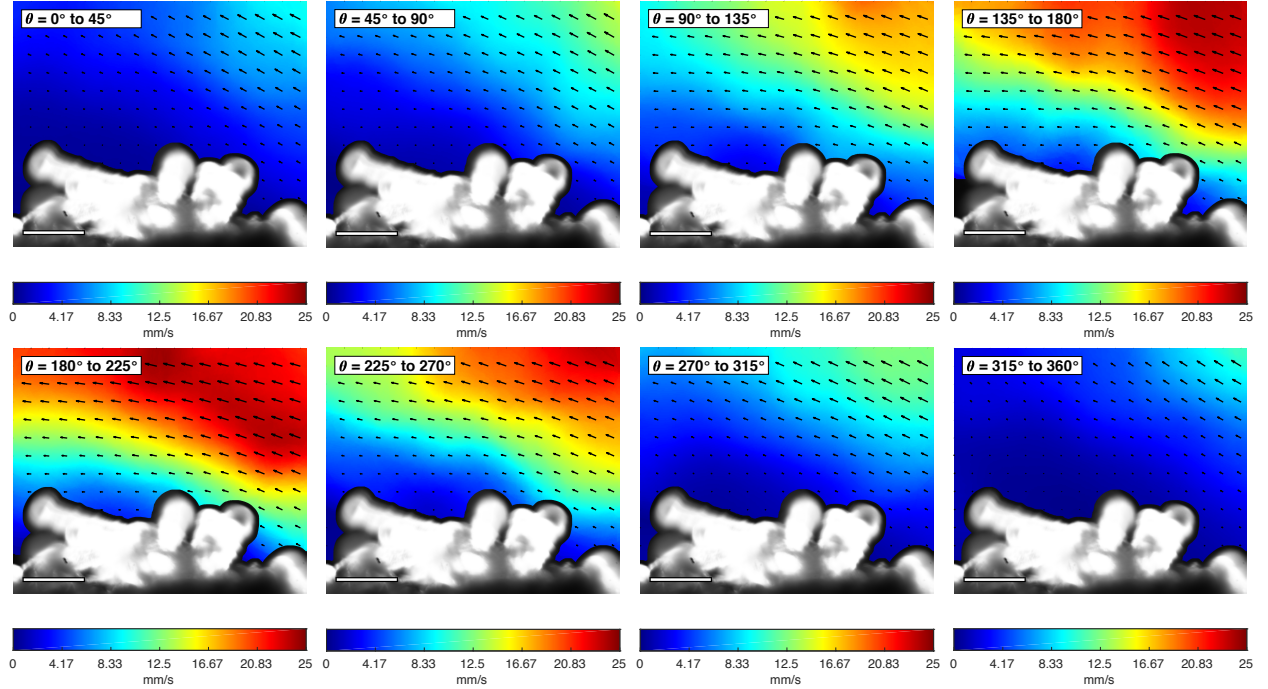


Figure 4.6 | Ensemble average velocity fields as a function of wave phase, Data Set #4.2. Two-dimensional velocity field calculated for each wave phase group using an ensemble average approach. Legend reports range of wave phases used in each ensemble.

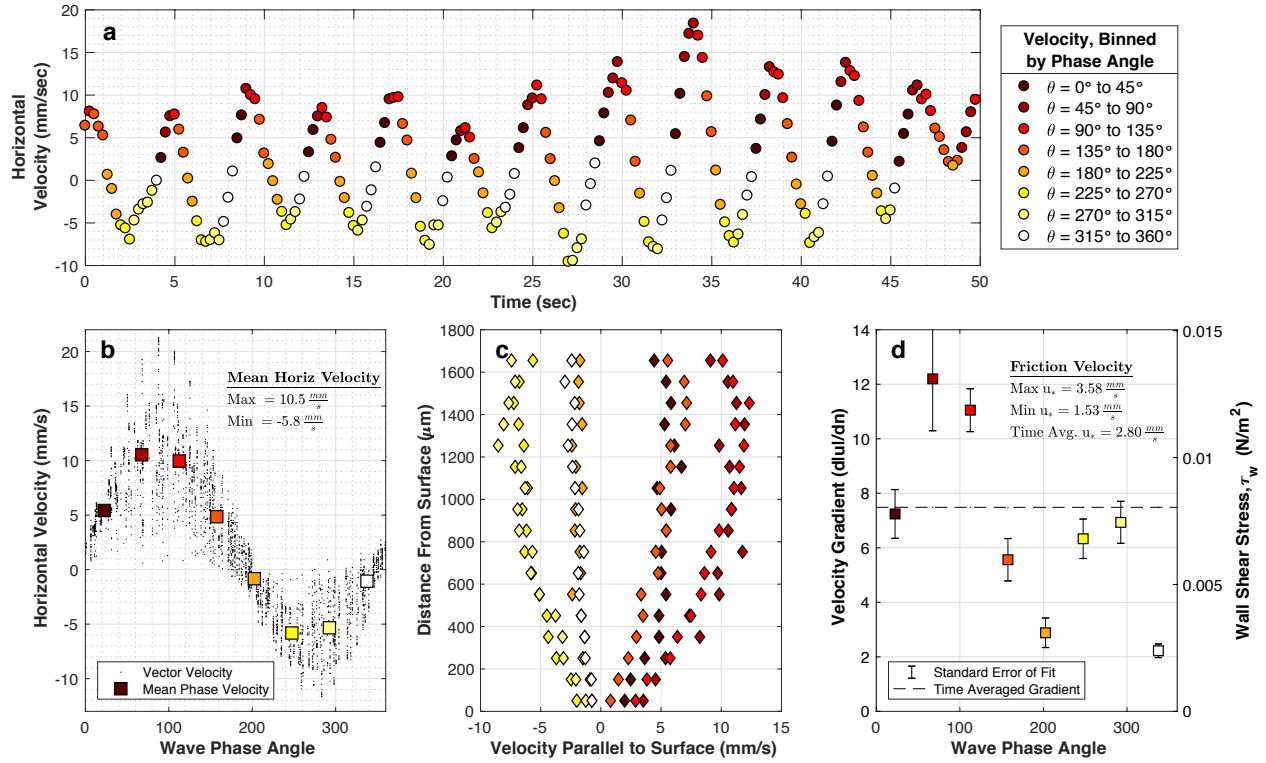


Figure 4.7 | Wave phase ensemble average, Data Set #4.1. All plots use the same color code for wave phase shown in the top right of the figure. (a) Mean horizontal velocity for each μPTV vector field as a function of time. A Hilbert transform was applied to this time series in order to determine an instantaneous phase for each vector field. Vector fields were then grouped into 8 wave phase groups as designated by the dot color. (b) Mean horizontal velocity as a function of phase. Small dots show horizontal velocities of each individual vectors in the data set. Large squares represent ensemble average velocity for each phase group. (c) Velocity profile determined for each wave phase group, vectors binned and a mean calculated every $100 \mu\text{m}$. (d) Velocity gradient for each phase group (determined using vectors from 0 to $600 \mu\text{m}$ from the surface). Dotted line shows the time-averaged velocity gradient calculated using all vectors in the data set. SE lines show standard error of the slope of the linear fit to the average velocity profile. Text reports friction velocities calculated based on the phase maximum, phase minimum, and time-averaged shear stress ($u_* = (\tau_w / \rho)^{1/2}$).

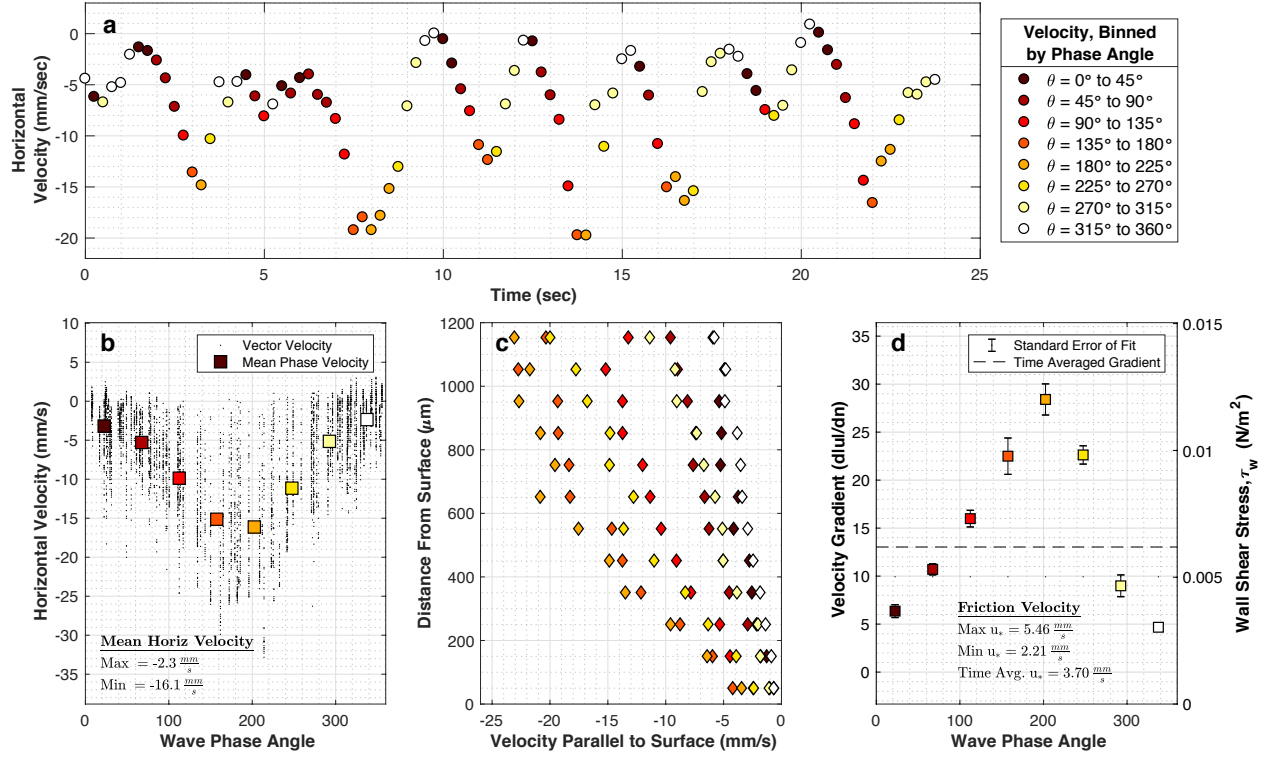


Figure 4.8 | Wave phase ensemble average, Data Set #4.2. All plots use the same color code for wave phase shown in the top right of the figure. (a) Mean horizontal velocity for each μ PTV vector field as a function of time. A Hilbert transform was applied to this time series in order to determine an instantaneous phase for each vector field. Vector fields were then grouped into 8 wave phase groups as designated by the dot color. (b) Mean horizontal velocity as a function of phase. Small dots show horizontal velocities of each individual vectors in the data set. Large squares represent ensemble average velocity for each phase group. (c) Velocity profile determined for each wave phase group, vectors binned and a mean calculated every 100 μ m. (d) Velocity gradient for each phase group (determined using vectors from 0 to 600 μ m from the surface). Dotted line shows the time-averaged velocity gradient calculated using all vectors in the data set. SE lines show standard error of the slope of the linear fit to the average velocity profile. Text reports friction velocities calculated based on the phase maximum, phase minimum, and time-averaged shear stress ($u_* = (\tau_w / \rho)^{1/2}$).

4.3.6 Velocity fluctuations, DBL thickness & mass transfer

Over the course of the ensemble average wave, peak velocities drive higher maximum shear stresses and corresponding higher friction velocities than time-averaged values (Fig. 4.6, Fig. 4.7). Each peak in near-surface shear is expected to compress the VBL and DBL to a minimum thickness as a result of more well mixed water coming closer to the surface^{55,56}.

Producing periodic increases in mass transfer. (Minimum DBL thickness can be approximated based on u_{*max} determined from phase averaging see Methods for details; Data Set #4.1:

$u_{*max} = 3.58 \text{ mm/s}$, $\delta_{DBL \min} \sim 290 \mu\text{m}$; Data Set #4.2: $u_{*max} = 5.46 \text{ mm/s}$,

$\delta_{DBL \min} \sim 190 \mu\text{m}$). The DBL thickness will then grow as fluid velocities and shear

subsequently decrease, before it is once again compressed by the next wave. In order to address the effects of these temporal dynamics on mass transfer we must consider the time-scale for the development of the DBL (t_{DBL}) and the frequency at which peaks in shear stress occur

($t_{peak \ shear}$)⁵⁵⁻⁵⁷.

The time scale for the DBL to fully develop can be approximated to a first order by the mean diffusion time through the DBL: $t_{DBL} \approx \delta_{DBL}^2 / D$, this follows previous work^{56,57} (where D is the rate of molecular diffusion, $D \approx 2 \times 10^{-5} \frac{\text{cm}^2}{\text{sec}}$)^{56,58}. Using this scaling $t_{DBL} \approx 20 \text{ sec}$ for $\delta_{DBL} = 200 \mu\text{m}$ (a lower bound on the DBL thickness observed here).

For Data Set #4.1: maximum peaks in shear occur with a period of $t_{peak \ shear} \approx 4.16 \text{ sec}$, for Data Set #4.2: $t_{peak \ shear} \approx 2.67 \text{ sec}$. For both data sets $t_{peak \ shear} < t_{DBL}$, as a result the DBL should not have time to fully develop between peak wave velocities⁵⁵. We therefore expect that the DBL thickness is controlled by the maximum shear stress at peak wave velocities and the frequency at which these peaks occur, rather than average water velocities or velocity magnitudes.

4.3.7 Spatial variability

μ PTV collects two-dimensional whole field measurements, this provides a means to examine spatial variability in fluid velocities and shear at the scale of individual coral polyps. The phase-averaged velocity fields (Fig. 4.5, 4.6) qualitatively show spatial variability in flow around the coral polyps.

In order to investigate this spatial variability further vector fields were vertically divided into two sections, and each half was then analyzed independently. The same ensemble averaging approach was applied that was previously used on the entire image. A phase average horizontal velocity and velocity gradient was calculated independently for each image half (Fig 4.9, 4.10). In Data Set #4.1, the coral polyp was contracted and the flow reversed in direction, this exhibited very low variation between the left and right sides of the polyp. In Data Set #4.2, the coral polyp was extended and the flow was unidirectional. Here the downstream side of the coral polyp exhibited reduced fluid velocities and velocity gradient. Additionally for Data Set #4.2, differences in shear between the two sides of the image increased with higher fluid velocities.

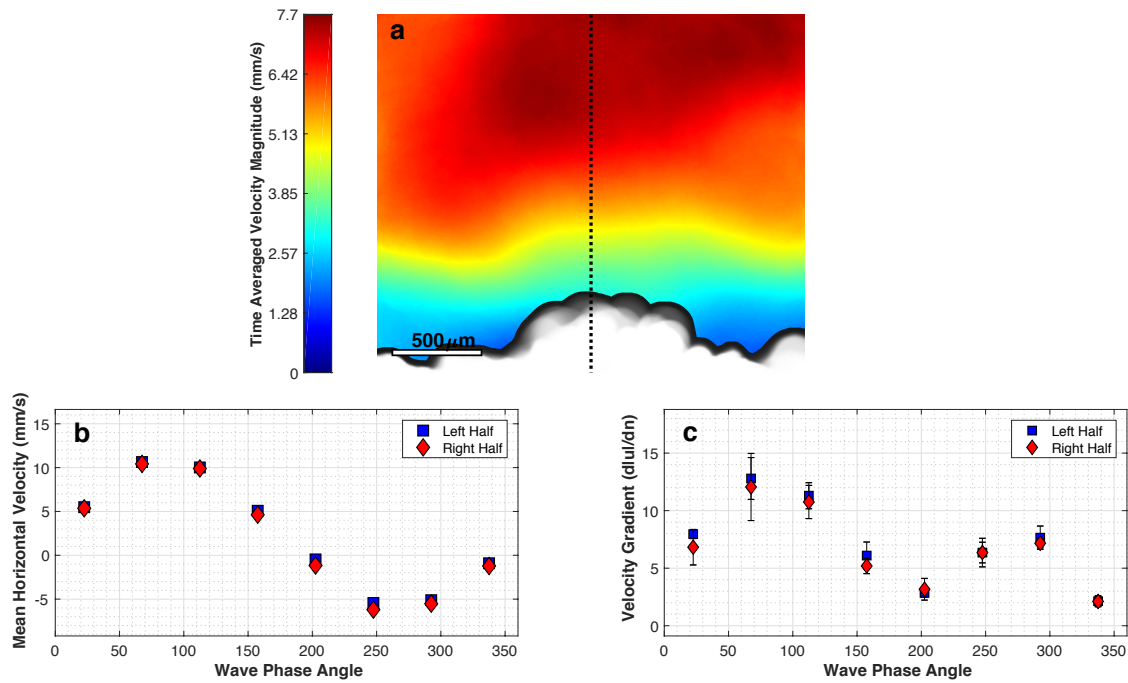


Figure 4.9 | Sectioned image, Data Set #4.1. (a) Time-averaged velocity magnitude, vertical line shows sectioning of image. (b) Phase-averaged horizontal velocity and (c) phase-averaged velocity gradient, calculated independently for each image half.

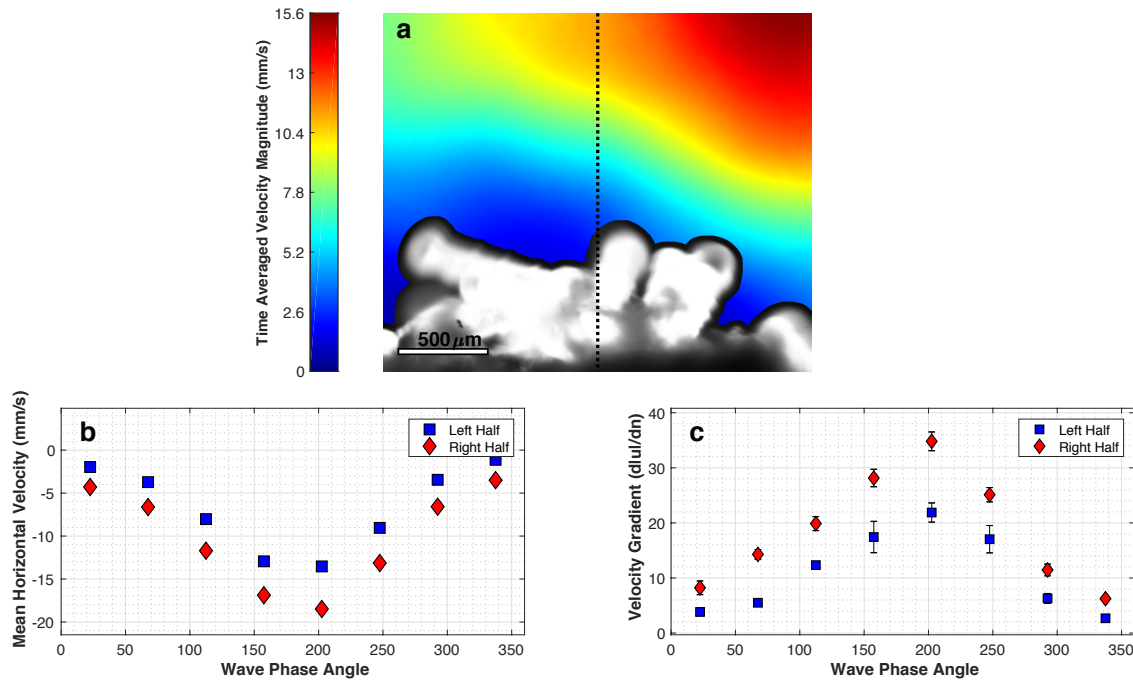


Figure 4.10 | Sectioned image, Data Set #4.2. (a) Time-averaged velocity magnitude, vertical line shows sectioning of image. (b) Phase-averaged horizontal velocity and (c) phase-averaged velocity gradient, calculated independently for each image half.

Another factor potentially impacting near-surface fluid dynamics is the direction at which surrounding flows approach the coral⁵⁹. Measurements of the coral DBL using microelectrodes have primarily been conducted in cases where water is flowing over the top of the coral colony and large-scale water motion is parallel to the coral's surface. However, as corals have significant three-dimensional structure, water may also be impinging on the vertical surfaces of the coral colony perpendicular to the large-scale flow^{31,60}. *In situ* measurements observed the same coral polyp experiencing parallel and impinging flows at different points in time (Fig. 4.11). These observations demonstrates that the local micro-flow environment may change significantly with time, and that the surrounding flow is not always parallel to the coral, and may be directed at an angle to the coral.

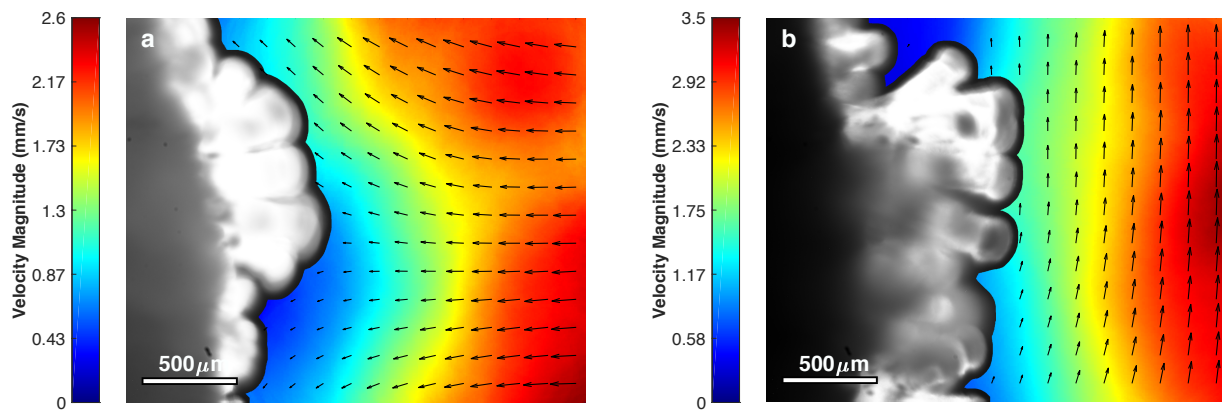


Figure 4.11 | Time-averaged velocity fields parallel and non-parallel to the coral's surface. (a,b) Images acquired *in situ* of the same coral polyp, approximately two days apart in time. Both figures are time series averages composed of 100 image pairs.

4.4 Discussion

In this study novel measurements were collected of spatiotemporal fluid dynamics at sub-millimeter scales around coral polyps in a natural reef environment. Temporal analysis of μ PTV measurements showed: 1) a linear VBL was present at the coral's surface *in situ*, 2) the near-surface velocity gradient was non-stationary and experienced strong periodic fluctuations, 3) velocity gradient fluctuations were driven by small amplitude ocean waves, 4) the DBL thickness was likely controlled by peak periodic velocities in the surrounding water rather than time-averaged currents. As compared to previous work these observations uniquely provided direct measurements of velocities within the VBL at the coral's surface *in situ*, and showed how dynamics in the VBL are influenced by the surrounding ocean environment.

Ecologically, these results show that small amplitude surface waves may have important impacts on mass transfer to and from corals, especially in low current environments. Peak velocities in the surrounding water periodically increased wall shear stress at the coral's surface – this is expected to depress the DBL, and based on time-scale analysis the DBL should not have had time to fully grow between waves. These findings indicate that under oscillating conditions peak velocities in the surrounding water may have a more impact on mass transfer and coral biological functions than average current strength. These analysis provide an important mechanistic perspective to previous studies at macro-scales which have shown enhanced coral mass transfer due to oscillatory wave motions^{30,61-63} as well as computational work showing high mass flux rates correspond to peak velocity magnitude in the surrounding water¹⁷. However, it is also important to recognize that μ PTV observations here only measured small-scale flows on the outer edge of the coral. Complex macro-scale flow through the colony^{31,60} may also be impacted by waves^{17,62,63}. Additionally the dynamics between fluctuating shear stress and the DBL

thickness are complex, they may vary for different flow conditions⁵⁶ and advection may also be important to consider⁵⁷.

Generalizing the results presented here, we must consider that wave orbital velocities attenuate with depth and shorter wavelength waves attenuate more rapidly. Therefore wave enhanced mixing due to oscillatory flows will have the strongest impacts on shallow corals and decrease with depth. Additionally wave and current strengths can vary dramatically for different reefs. Other coral reefs, such as those with strong circulation⁶⁴, can have much high water velocities than Eilat which is protected in a long narrow gulf. This relatively low flow environment is the type of setting where the DBL is likely to be thicker and as a result have a more significant impact on corals. Oscillatory water motions may be especially important for such locations with comparatively low currents.

There is significant potential to build on the investigation presented here. By collecting simultaneous ADV and μ PTV measurements under a range of flow conditions, it might be possible to develop calibrations relating the two measurements. Such that near surface velocities can be estimated based on surrounding flows. Time series observations also offer a means to investigate how the DBL may be effected by quasi-periodic turbulent sweep and ejection events that cause surface renewal^{57,65-67}.

Future work using both μ PTV and chemical micro-electrode measurements³⁸ performed in lab flumes or *in situ* may be very informative. Micro-electrode measurements of chemical concentration have shown the presence of a coral DBL, that its thickness varies inversely with flow speeds in flumes^{11,68}, and that it can vary spatially over coral polyp structure^{12,69}. However, micro-electrode observations provide only a point measurement, do not measure fluid velocities, and have only rarely measured the *in situ* coral DBL^{15,70}. The results here also suggest that lab

studies of corals using micro-electrodes should incorporate oscillating flows, as existing studies have most often been conducted under steady water motion^{11,12}.

In addition to temporal dynamics μ PTV measurements also allowed spatial analysis. In this study, observations showed the flow field can vary with local roughness at the polyp scale. A decrease in velocity and shear was observed between the up and downstream sides of a polyp, potentially indicating the presence of a small wake region developing downstream of the polyp⁷¹. Impacts of polyp structure and flow are likely to vary as a function of coral polyp extension as well as the direction of surrounding water motion, and are worthy of future investigation. Many corals expand and contract their polyps diurnally⁷². This dramatically changes their exposed surface area as well as their small-scale roughness, both of which may impact the rates of mass exchange with the surrounding water. Several studies have considered the impacts of coral polyp extension and small-scale roughness on mass transfer, with varying conclusions^{3,34,49,61,73,74}. Additionally small-scale roughness over marine sediments has been found to have some, relatively small, impacts on mass transfer rates^{10,75}. μ PTV offers a potentially powerful mean for conducting mechanistic investigations on the impact of coral polyp extension and contraction as well as morphology on near-surface flows and chemical fluxes^{3,49,73,74}.

Previous lab observations of coral fragments in milli-fluidic chambers found that coral cilia actively mix water at the coral's surface³⁸. During μ PTV testing in aquariums we also observed what appeared to be cilia driven water motion at the coral's surface. However thus far we have not clearly observed any coral cilia driven flows *in situ*. This may be due to ocean currents and waves overwhelming cilia flows. If this is the case, cilia generated mixing may still be important for low flow regions on the coral's surface such as between extended polyps or on branches near center of colony, although this was not observed or investigated here.

Small-scale water motions at the coral-water interface influence coral health and basic biological processes. Using direct *in situ* observations at previously unresolved scales, this study has shown how ocean waves can impact near-surface fluid dynamics. This work additionally demonstrates the importance and utility of conducting direct *in situ* observations of the benthic marine organisms and their surrounding environments at micro-scales.

4.5 Methods

4.5.1 Underwater micro-particle tracking velocimetry

This study utilizes a new underwater μ PTV instrument, capable of measuring two-dimensional velocity fields at sub-millimeter scales directly next to surfaces *in situ*. The μ PTV instrument uses a diver operated underwater microscopic imaging system equipped with a long working distance telecentric objective lens⁷⁶. This is augmented with dark field transmission illumination, rapid image pair acquisition using frame straddling, and active seeding of tracer particles (10 μ m hollow glass spheres, inert and neutrally buoyant).

The system images a field of view of approximately 2.5 x 2.1 mm, while maintaining a working distance of approximately 95 mm from the imaged volume to both the imaging and illumination units. For analysis applied here, 90% of particles are detected within approximately +/- 770 μ m from the most in-focus plane. (Calibrations shown in Supplementary Fig. 4.8; the thickness of this detection plane can be adjusted based on the particle detection algorithm, with a thicker detection plane resulting in more identified particles. The same tracking parameters were used for all analyses performed here.) Short illumination pulses of 100 μ s or less produced low particle blur. The system recorded 4 frame pairs per second during the field deployments described here (it is capable of up to 7 frame pairs per second).

In post processing individual particles are identified and matched between images to produce a two-dimensional field of two-component velocities vectors. All processing was conducted in Matlab using established PTV techniques. First images are spatially bandpass filtered. Next, particles are identified using code based on the widely used IDL tracker⁷⁷. Particles are then matched between images using a non-iterative approach constrained by neighboring particle displacements⁷⁸. Finally, global thresholding was applied to eliminate spurious vectors based on vector magnitude and angle. Images also capture the polyp's state (open or closed) and location of the polyps surface.

4.5.2 Field deployment

Underwater μ PTV data was collected on a coral reef offshore of the Interuniversity Institute for Marine Sciences (IUI) in the Gulf of Eilat. This is a long, narrow gulf (approximately 160 x 24 km) off the Red Sea. This reef typically experiences relatively small amplitude wind waves and low currents on the order of 5-10 cm/s.^{47,48}

The μ PTV system was deployed by a team of two scientific divers on a sandy sloping shelf at approximately 5 m depth and 35 m from shore, near the end of the IUI pier. There it was placed around a small isolated coral colony of *Pocillopora spp.* (colony diameter approximately 10 cm), and positioned to image coral polyps near the colony edge. During *in situ* operations the μ PTV system was fully controlled by divers using an underwater computer. Following deployment control and data collection was conducted from shore using a data and power cable routed to the IUI pier, which provided a live image feed to the instrument.

An ADV (Nortek Vector) was placed next to the coral approximately 15 cm away from the volume imaged by the μ PTV system and approximately 10 cm off the seafloor (roughly the

same height as the point imaged by the μ PTV system). The ADV operated at 16 Hz measuring x, y, and z fluid velocities in an approximately 3.5 cm^3 volume, as well as recording pressure. The ADV time series data was convolved with a 1 second Gaussian filter in order to reduce noise.

4.5.3 Average velocity fields

Two-dimensional time-averaged and phase-averaged velocity fields were calculated by combining many vectors fields. The velocity at each point in the two-dimensional field-of-view was calculated using a weighted average of all vectors from the data set within a $400 \text{ }\mu\text{m}$ radius. The average was Gaussian weighted such that vectors closer to the point of interest contributed more strongly than vectors further away, following the method described by Agüera et. al. 2016⁷⁹. For time-averaged flows (in which fluid velocities may reverse direction) absolute magnitude of velocity was used and as a result directional information is not included in the image.

4.5.4 Shear stress & friction velocity

The velocity gradient within the linear VBL region at the surface-water interface can be used as a direct measure of wall shear stress: $\tau_w = \mu \frac{\partial \bar{u}}{\partial n} \approx \mu \frac{d\bar{u}}{dn}$ (where $\mu = \nu \cdot \rho$ is the dynamic viscosity of the fluid, $\bar{u}$ is the average velocity parallel to the surface, and n is distance from the surface), and friction velocity: $u_* = (\tau_w / \rho)^{1/2}$. This approach has previously been applied to a variety of velocity measurement including μ PTV observations of flow over mechanical surfaces in the lab^{7,8} and flow within blood vessels *in vivo*^{41,42}, as well as one dimensional velocity profiles at the ocean floor⁸⁰⁻⁸². The velocity gradient was defined as the change in velocity component parallel to the surface as a function of distance from the surface: $\frac{d\bar{u}}{dn}$ (where $\bar{u}$ is the

average velocity parallel to the surface, and n is distance from the surface)⁴³. The distance of each vector to the coral's surface was calculated, as well as the component of each velocity vector perpendicular to the local coral surface normal vector.

For time series and phase ensemble averages of many vector fields, the average velocity gradient was determined by first grouping all vectors from the data set in bins every 100 μm from the surface. An average velocity was then calculated for each bin, and an ordinary least squares linear fit was applied to these average velocities. The linear fit was applied to the first six bins (i.e. 0 to 600 μm from the surface) as this region demonstrated the most linear gradient. The coefficient of determination (r^2) and standard error of the slope (SE) of the linear fit were then calculated. This binning approach and determination of the standard error of the slope follow the methods applied in previous work^{8,83}.

For instantaneous velocity gradients of single vector fields, a least squares fit was applied directly to the vectors and the fit was applied over the entire field-of-view (rather than only 0 to 600 μm). This approach was used because some image pairs produced relatively sparse vector fields. The instantaneous velocity gradients show a slightly reduced slope as compared to the phase-averaged gradients which only use velocities from 0 to 600 μm from the surface.

It is important to note that the system does not measure out-of-plane velocities. As a result shear stress may be greater than measured by the μPTV system if out-of-plane velocities are significant. The measurements here should thus be considered as lower bounds.

4.5.5 DBL scaling

The DBL thickness can be estimated from the VBL thickness or friction velocity using scaling relationships. This scaling can be performed using the Schmidt number, which is a ratio

of momentum diffusion (kinematic viscosity) to molecular diffusion ($Sc = \frac{\nu}{D}$, ν is kinematic viscosity and D is molecular diffusion). For dissolved gases in water at ambient temperatures, Schmidt numbers typically range from approximately 500 to 1,000.⁹ Mass transfer theory for steady flow over a flat surface predicts that: $\frac{\delta_{VBL}}{\delta_{DBL}} = Sc^{\frac{1}{3}}$, and for Schmidt numbers of interest here: $\delta_{DBL} \approx \frac{\delta_{VBL}}{10}$.^{3,9} DBL thickness can also be expressed directly as a function of friction velocity using the equation: $\delta_{DBL} \approx \delta_{VBL} Sc^{-\frac{1}{3}} \approx 10 \frac{\nu}{u_*} Sc^{-\frac{1}{3}}$.^{9,18,44} This scaling has been validated experimentally for steady flow over sediments, although the leading constant may vary slightly^{18,44}.

Wave action observed in this study as well as the intricate coral structure may make this relationship more complex. However simulations of oscillating flow over corals have shown local shear stress is still a proxy for mass transfer¹⁷. Here we use the simple relationship $\delta_{DBL} \sim 10 \frac{\nu}{u_*} Sc^{-\frac{1}{3}}$ (where $Sc=1,000$) to approximate DBL thickness based on friction velocity – due to the system's complexity, as well as our inability to observe out-of-plane flows, we state this DBL thickness as only an order-of-magnitude approximation. Important conclusions in this study relate primarily to how expected DBL thickness is modulated, rather than its absolute value.

4.5.6 Wavelength & wave amplitude calculations

The wavelength of the ocean surface waves modulating the system was calculated from the water depth and the wave period as measured by the ADV and μ PTV instruments. Assuming linear wave theory applies, the relationship between these parameters can be described by the dispersion equation: $\omega = \sqrt{gk \tanh(kh)}$, (where g is acceleration due to gravity; $\omega = \frac{2\pi}{T}$ is

wave angular frequency; T is wave period; h is the depth of the seafloor; $k = \frac{2\pi}{L}$ is the wave number; and L is wavelength)^{45,46}.

The wave height was also estimate based on sub-surface pressure measurements recorded by the ADV^{45,46}. For linear waves, the sub-surface pressure is a function of three parameters: wave frequency, seafloor depth, and pressure sensor depth. Assuming linear wave theory applies, subsurface pressure follows the equation: $p = -\rho g z + \rho g \eta K_p$, (where ρ is density; g is acceleration due to gravity; z is distance from the free surface to the sensor, positive upward; η is the displacement of the water surface from the mean; K_p is the pressure transfer function)⁴⁶. Pressure fluctuations due to waves attenuate from the surface to depth, additionally high frequency waves attenuate faster than low frequency waves. The pressure transfer function is defined as: $K_p = \frac{\cosh(k(h+z))}{\cosh(kh)}$ (variables following previous definitions)^{45,46,84}. Here the pressure sensor is very close to the seafloor such that: $K_p \approx \frac{1}{\cosh(kh)}$.

In order to estimate fluctuations in water surface, subsurface pressure is first converted to pressure head: $H_p = \frac{p}{\rho g}$ which has units of meters. The subsurface pressure head time series is then converted to a power spectral density (PSD) using Fourier analysis, this represents the distribution of energy as a function of frequency. Next the PSD of sub-surface pressure is used to estimate a PSD of surface elevation by applying the pressure transfer function: $S_{\eta\eta}(f) = \left(\frac{1}{K_p^2}\right) S_{pp}(f)$, (where $S_{pp}(f)$ is the PSD of subsurface pressure; and $S_{\eta\eta}(f)$ is PSD surface elevation). This approach follows previous work^{45,46,84}, and is implemented in Matlab based on open source code⁸⁵ (Supplementary Fig. 4.4, 4.5). The surface wave spectra is then used to estimate significant wave height, H_s , which is the mean wave height of the highest one third of

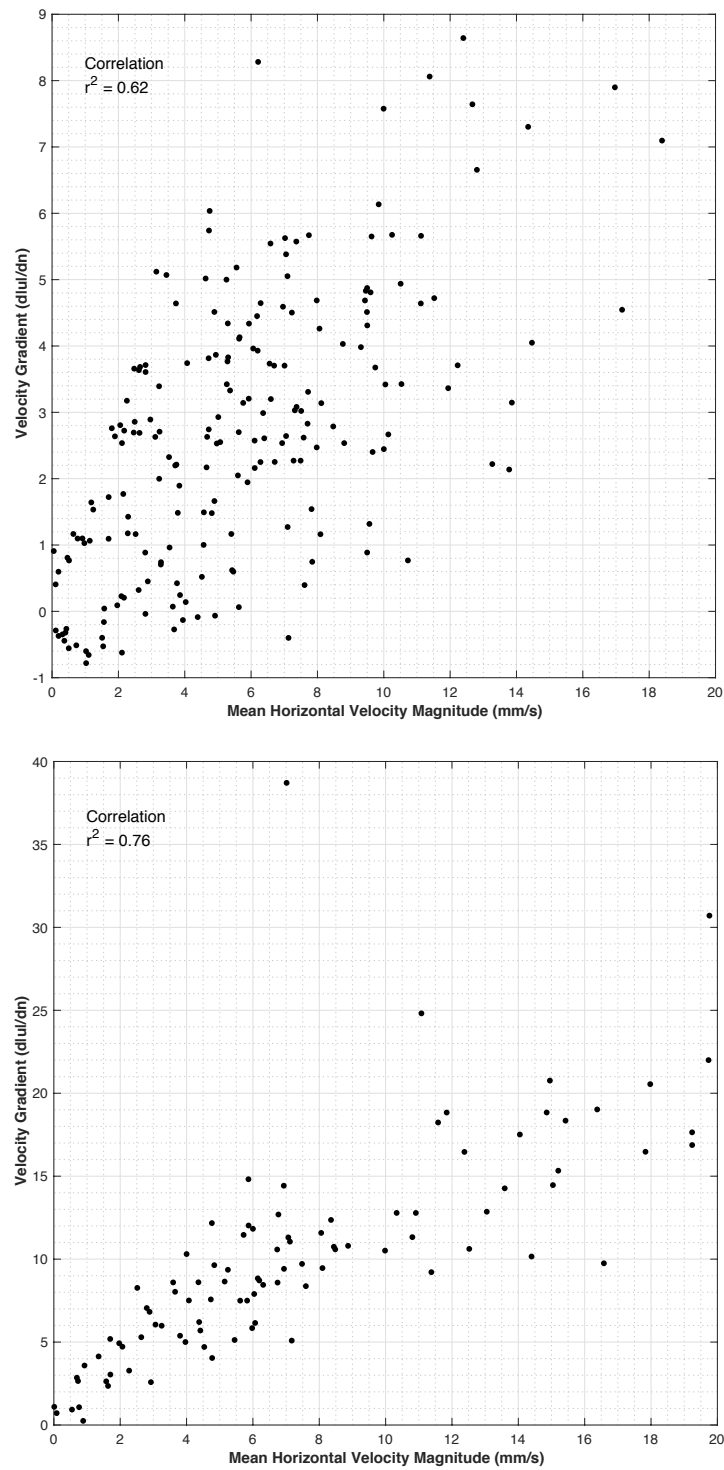
waves. This can alternatively be defined: $H_s = 4\sqrt{m_0}$, (where m_0 is the zeroth-order moment of the wave spectrum)^{46,85}.

These predictions of surface wave height can then be checked by using wave height to estimate orbital wave velocities at the seafloor⁸⁶, and comparing these estimates to the ADV velocity measurements.

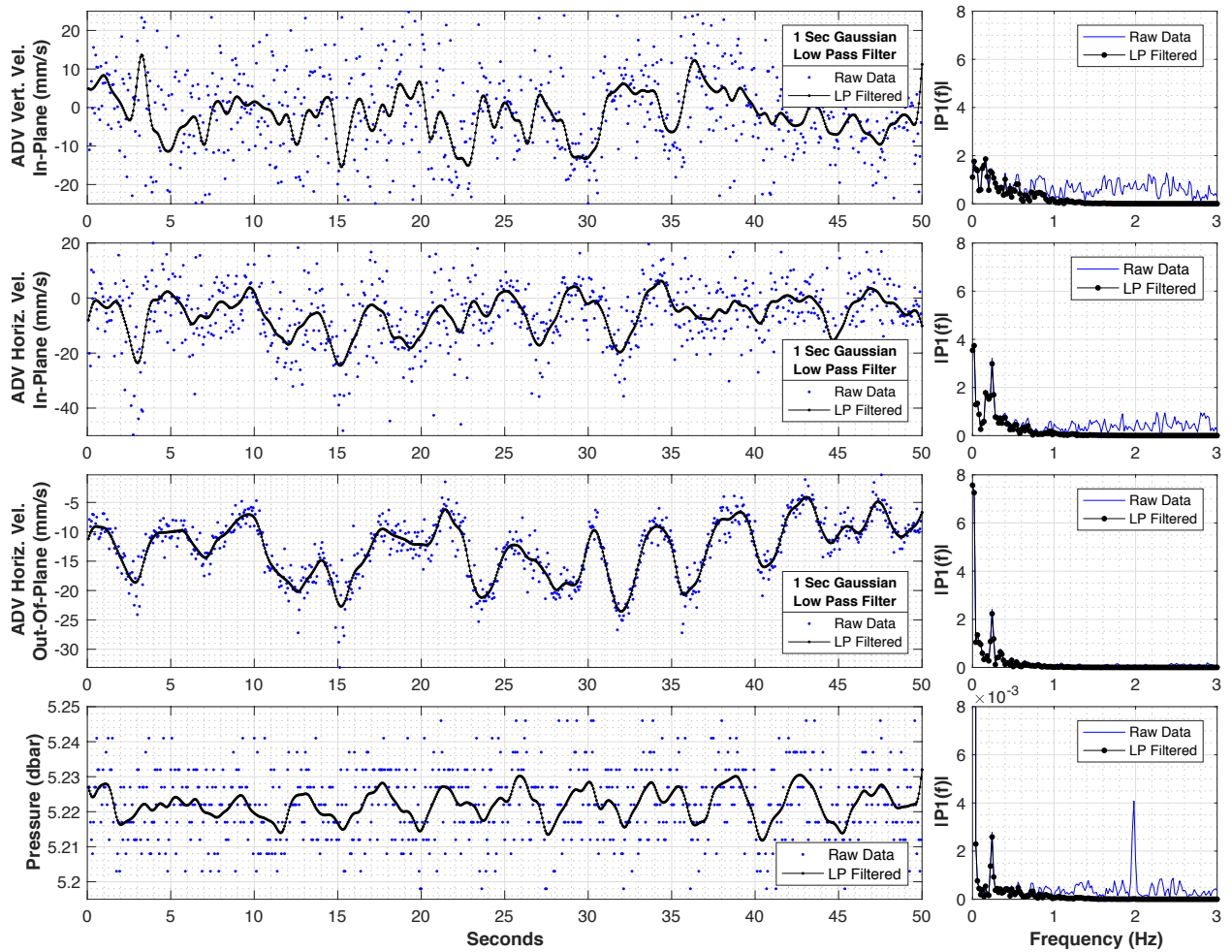
4.5.8 Data selection

During *in situ* instrument deployments we collected a large number of images. Two time series image sets were selected for the detailed analysis presented here (Data Set #4.1 and Data Set #4.2). These were selected as they had relatively high particle counts throughout the time series, demonstrated clear and distinct wave oscillations, and were recorded during simultaneous ADV measurements.

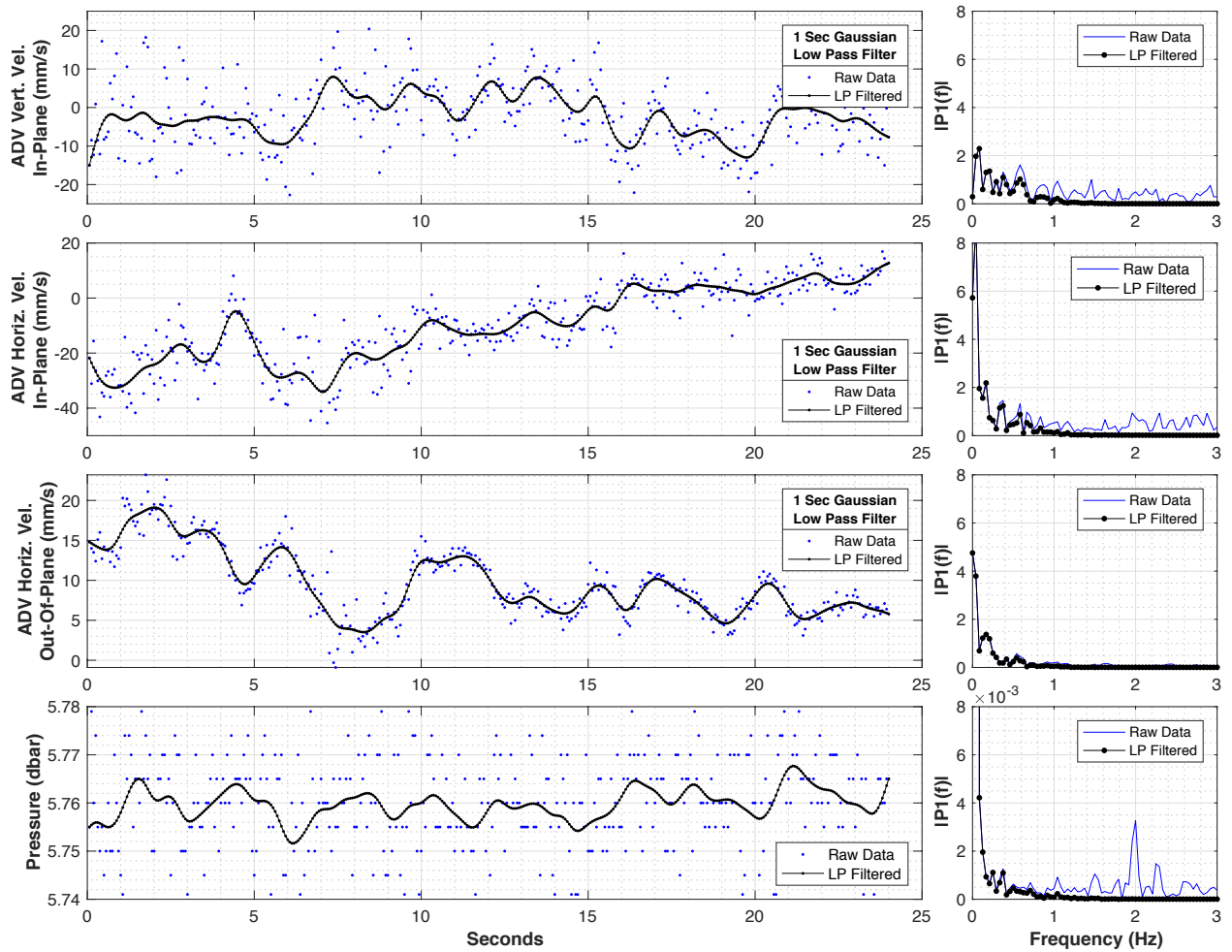
4.6 Supplementary



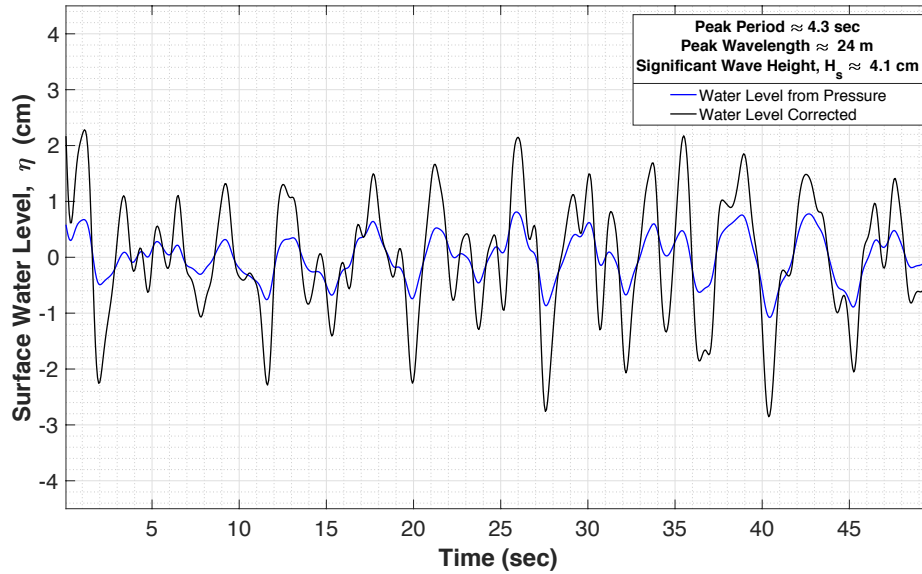
Supplementary Figure 4.1 | Correlation of instantaneous horizontal velocity magnitude and instantaneous velocity gradient. (a) Data Set #4.1, polyps contracted, (b) Data Set #4.2, polyps expanded



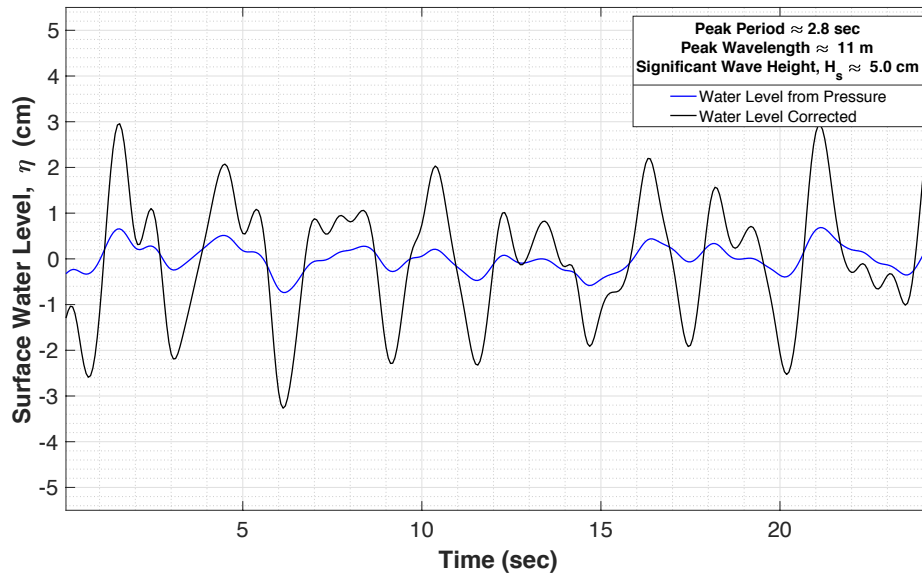
Supplementary Figure 4.2 | ADV data corresponding to Data Set #4.1. Graph shows original raw data (blue) and data after low pass filtering (black). Low pass filtering was performed by convolving time series with 1 sec Gaussian function.



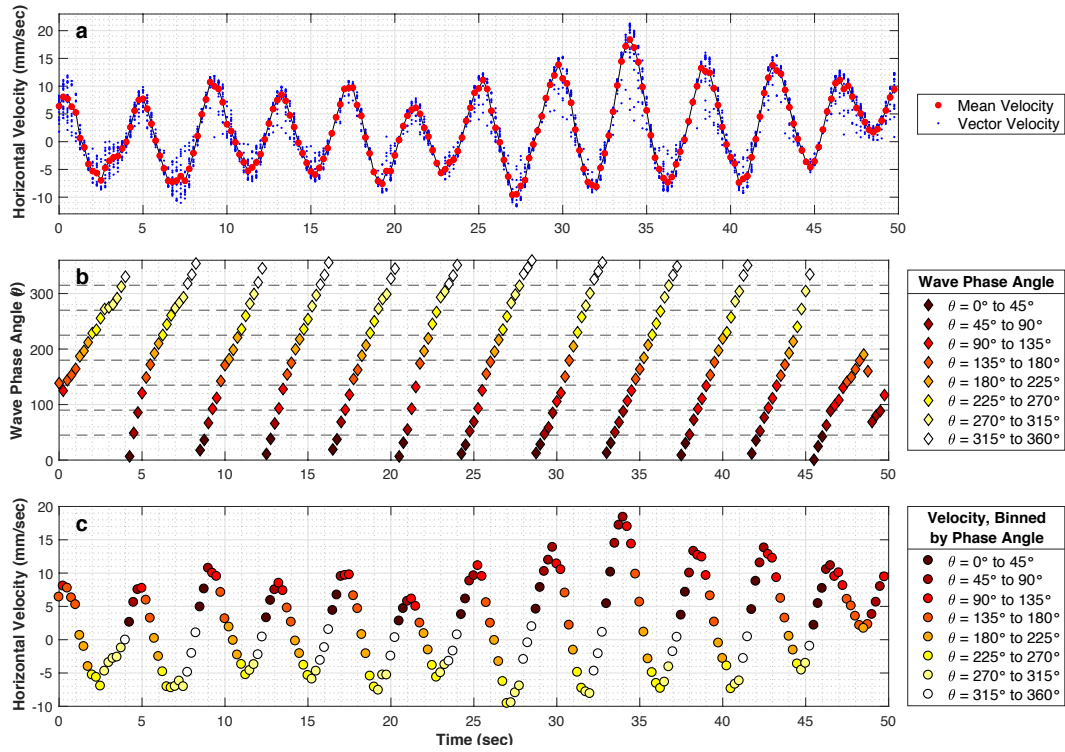
Supplementary Figure 4.3 | ADV data corresponding to Data Set #4.2. Graph shows original raw data (blue) and data after low pass filtering (black). Low pass filtering was performed by convolving time series with 1 sec Gaussian function.



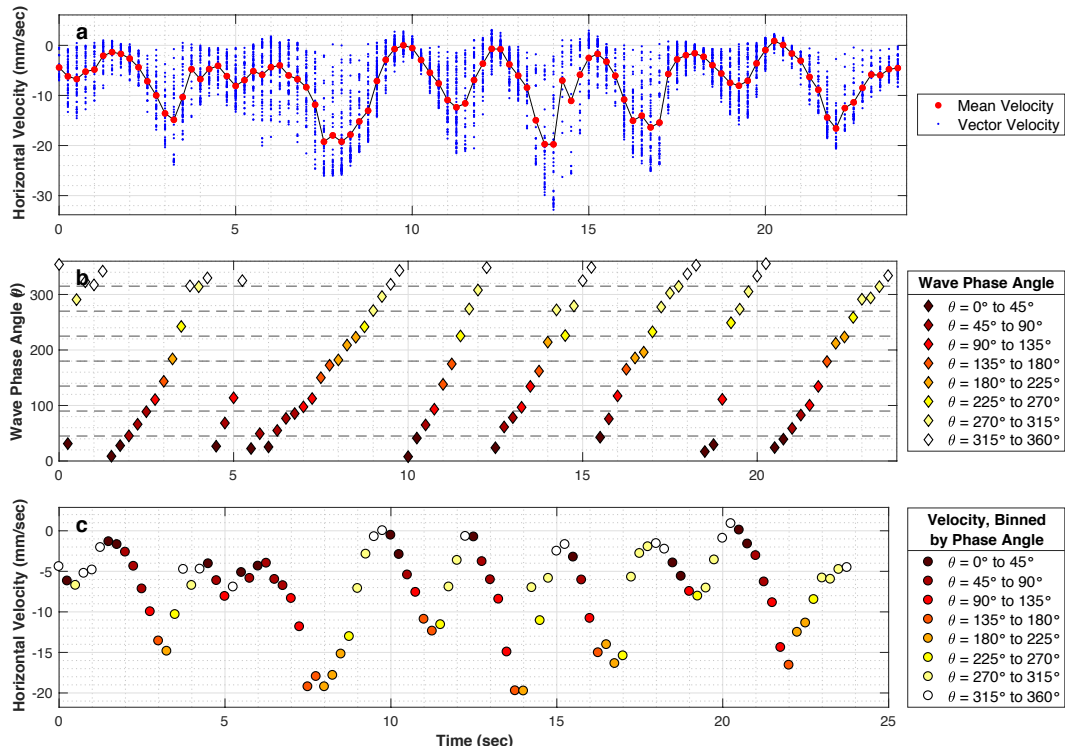
Supplementary Figure 4.4 | Data Set #4.1, water surface elevation calculated from ADV pressure measurement. Blue line shows surface elevation calculated directly from pressure, black line shows surface elevation calculated from pressure and corrected using pressure transfer function. Legend reports the dominant the wave period, as well as wavelength calculated from the wave period using linear wave theory. Additionally significant wave height (highest third of waves) is reported based on retrieved water surface level.



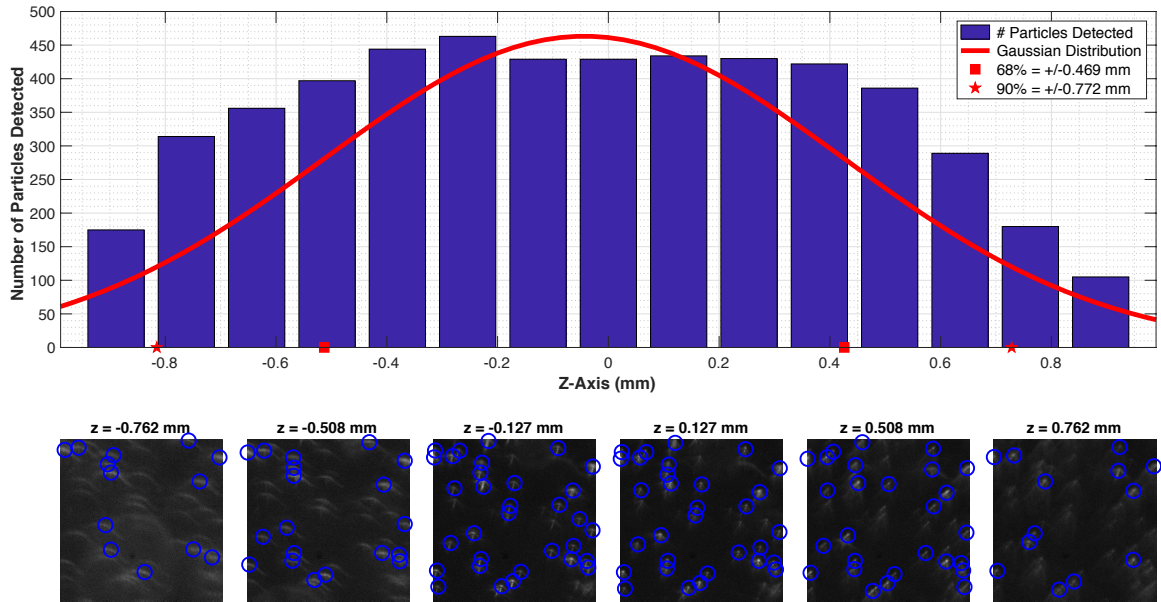
Supplementary Figure 4.5 | Data Set #4.2, water surface elevation calculated from ADV pressure measurement. Blue line shows surface elevation calculated directly from pressure, black line shows surface elevation calculated from pressure and corrected using pressure transfer function. Legend reports the dominant the wave period, as well as wavelength calculated from the wave period using linear wave theory. Additionally significant wave height (highest third of waves) is reported based on retrieved water surface level.



Supplementary Figure 4.6 | Phase binning using Hilbert transform, Data Set #4.1.



Supplementary Figure 4.7 | Phase binning using Hilbert transform, Data Set #4.2.



Supplementary Figure 4.8 | Measurement plane width calibration. Calibration for depth of measurement plane The thickness of the measurement plane (the volume over which the μ PTV system detects particles), was calibrated by imaging a microscope slide with many particles in the lab flume testing, and translating this slide in the z-direction. The top graph shows the number of particles detected as a function of depth, a Gaussian curve was then fit to this distribution to estimate the thickness of the plane over which particles were are detected. The bottom graph shows a sub-sample of the field of particle field of view (500x500 pixels) at several different z-positions, with detected particles circled in blue.

Supplementary Video 4.1 | μ PTV time series, Data Set #4.1. Left side: Vector field for each μ PTV image pair. Right side: Corresponding polar plot for vectors, each blue dot represents one vector. Distance from center of polar plot is proportional to velocity magnitude in mm/s, polar plot angle matches vector angle.

Supplementary Video 4.2 | μ PTV time series, Data Set #4.2. Left side: Vector field for each μ PTV image pair. Right side: Corresponding polar plot for vectors, each blue dot represents one vector. Distance from center of polar plot is proportional to velocity magnitude in mm/s, polar plot angle matches vector angle.

Supplementary Video 4.3 | Wave phase ensemble average, Data Set #4.1. Bottom: Average horizontal velocity for vector field time series. Points are color coded by their phase group (determined using a Hilbert transform) and all time points in the current phase group are circled in black. Top Left: Two-dimensional ensemble average velocity field. Velocity magnitude at each point is determined by a Gaussian weighted average of all vectors in the phase group within 400 μ m. Top Right: Velocity profile as a function of distance from the surface. Blue dots represent individual vectors, red dots represent velocities averaged every 100 μ m from the surface. Linear fit applied to binned velocity averages (red dots) from 0 to 600 μ m from the surface. Linear fit parameters are displayed on the video. Video repeats the same loop 10 times.

Supplementary Video 4.4 | Wave phase ensemble average, Data Set #4.2. Bottom: Average horizontal velocity for vector field time series. Points are color coded by their phase group (determined using a Hilbert transform) and all time points in the current phase group are circled in black. Top Left: Two-dimensional ensemble average velocity field. Velocity magnitude at each point is determined by a Gaussian weighted average of all vectors in the phase group within 400 μ m. Top Right: Velocity profile as a function of distance from the surface. Blue dots represent individual vectors, red dots represent velocities averaged every 100 μ m from the surface. Linear fit applied to binned velocity averages (red dots) from 0 to 600 μ m from the surface. Linear fit parameters are displayed on the video. (and does not consider DBL temporal dynamics). Video repeats the same loop 10 times.

4.6 Acknowledgements

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Chapter 4, in part, is currently being prepared for publication: A.D. Mullen, A. Genin, P.L.D. Roberts, J.S. Jaffe “*In Situ* Micro-Scale Fluid Dynamics at the Coral-Ocean Interface.” The dissertation author was the primary investigator and author of this material.

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