Cytometers set sail with sea-going mobile robots

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Marine microbial communities play a critical role in Earth's biosphere. Phytoplankton produces at least 50% of breathable oxygen, regulate atmospheric CO_2, and form the base of the marine food web. Toxic algal blooms have major effects on coastal ecosystems and fisheries as well as human health. Knowledge of phytoplankton distribution, diversity and abundance in the ocean is largely due to instruments called flow cytometers (Figure 1), which produce quantitative multi-parametric measurements of individual particles at rates of thousands per second or more (Shapiro, 2003). Measurements made using cytometric parameters such as light scatter and fluorescence can reveal cell size and intrinsic pigments, which are useful in characterizing plankton taxonomy, abundance and viability (Figure 2). Most cytometric measurements are made from ships, buoys, and cable-to-shore observatories. Integration of cytometers into autonomous surface and underwater vehicles could greatly advance marine microbiology by enabling better spatial and temporal sampling, facilitate faster "event response", could provide access to climate-sensitive polar under-ice regions, and may lower costs relative to ship operations. In this article we discuss instrument and vehicle development trends that point the way to these mobile robot cytometers.

FIGURE 1

Generic diagram of a flow cytometer. Particles for analysis are introduced into a nozzle or cuvette for analysis, where hydrodynamic focusing produces a single-file flow of particles past a focused laser. Light scatter and fluorescence measurements are made by photomultiplier tubes (PMTs) detecting selected wavelengths as determined by optical bandpass filters. PMT output is converted by analog to digital converters (ADCs) for computer display of particle intensity levels. Image used with permission, Semrock.com

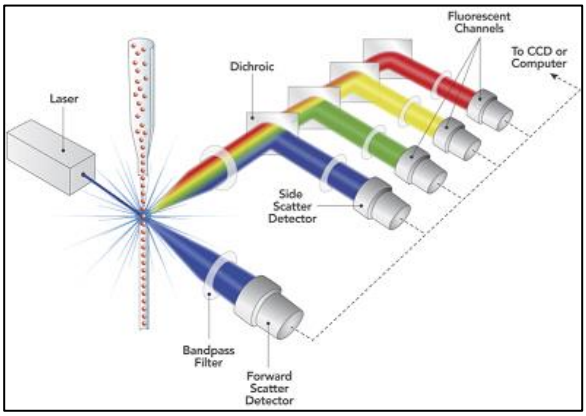
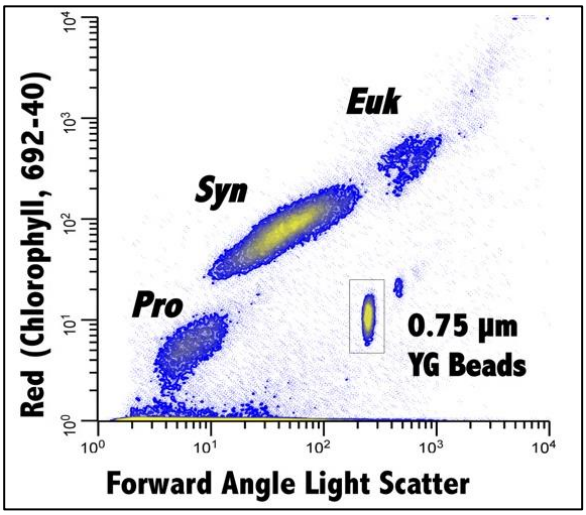


FIGURE 2

Flow cytometric analysis of plankton. Levels of 692nm chlorophyll fluorescence plotted against forward slight scatter intensity allow discrimination of various species, *Prochlorococcus* (Pro), *Synechococcus* (Syn), and eukaryotes (Euk). Sample is spiked with 0.75micrometer yellow green "YG" beads (Polysciences, Inc.) as a size reference. Image courtesy of Amy Zimmerman, MBARI



Long-duration untethered autonomous vehicles complement other data gathering platforms such as research vessels and moorings since they can sample broad areas and volumes, and have proven themselves revolutionary in the ocean sciences (Bellingham, 2014). The inclusion of cytometers on these vehicles would greatly advance marine microbiology, but this integration presents challenges. Existing vehicles have limited size and power budget that must be considered. Traditional flow cytometer designs offer unique challenges for autonomous vehicle integration. The typical hydrodynamic focusing utilized for optimal positioning of particles in the flow stream requires a source of particle-free “sheath” fluid. Laser light sources, focusing optics and detection systems must maintain precise alignment. In addition many desirable flow cytometric assays such as total DNA or viability assessment require processing of material before entering the flow cytometer for analysis (Veldhuis and Kraay, 2000, Lomas et al, 2011). Data processing and communication capabilities add another constraint to cytometer integration. Clearly integration of flow cytometers as AUV payloads would benefit from optimized cytometer design.

A consideration of the minimum number of flow cytometric parameters that can provide reliable characterization of plankton is important for producing a focused instrument design. The fact that many species of plankton can be identified by cytometric measurement of the intrinsic qualities of the organism, such as light scatter (approximate size) or fluorescence from native pigments, helps to reduce the parameters needed (Sosik et al, 2010). In addition, novel approaches to the traditional flow cytometer design can further minimize the size/power footprint of an instrument destined for a vehicle. Instruments that simplify sample handling by measuring intrinsic phytoplankton fluorescence without the need for staining cells beforehand or sorting them in the cytometer are the best candidates for early integration.

Parameters that have proven beneficial for marine application of cytometers include Coulter volume, light scatter and fluorescence, time-of-flight pulse shape characterization, and imaging techniques. Some instruments have been developed and deployed that combine some of these techniques; the CytoBuoy (CytoBuoy b.v., Woerden, the Netherlands) for pulse shape and image capture, and the Imaging Flow CytoBot (WHOI, Woods Hole, MA) adds image analysis. The wide range of sizes (0.2-200 micrometers) and shapes (round, rod-like, chains) of marine phytoplankton adds a daunting challenge in designing one cytometric system that can accurately identify all types of plankton. Traditional laboratory based cytometers have not been able to analyze such a large dynamic range of particle sizes in one instrument without adding complexity to the system (Beckman Coulter Astrios EQ), or

designing systems specific to a portion of the dynamic range (Apogee A50).

<<H1>>STATE OF THE ART

<<H2>>Autonomous vehicle benefits and constraints

Autonomous surface vehicles (ASVs) are robot boats that move along the sea surface, and may be equipped with profilers for acquiring data at depth. Autonomous underwater vehicles (AUVs) move in three dimensions and may be propeller driven, or buoyancy driven in the case of gliders. Autonomous vehicles provide a number of benefits compare to manned vessels:

Automated surveys: Autonomous vehicles can be dispatched to a specific location, and underwater vehicles, to specific depths, enabling 2D and 3D surveys. The survey may be commanded by a human operator or another machine, or the vehicle itself may alter survey patterns in response to *in situ* sensor data.

Operational cost/scalability: As a point of comparison, a *Wave Glider* ASV (Liquid Robotics, Sunnyvale, CA) costs MBARI about $900/day to operate; while MBARI's 41-meter *RV Rachel Carson* costs about $8,000/day. Due to lower cost of equipment and operation, multiple autonomous vehicles running multiple missions can be used at lower cost than one research vessel.

Endurance: Some autonomous vehicles are capable of long deployments lasting weeks or even months, spanning thousands of kilometers; for example the *AutoSub LR* (Furlong et al, 2012), the *LRAUV* (Hobson et al, 2012), and the *Spray* glider (Davis et al, 2002). These vehicles enable continuous presence in a region of interest, provide opportunities to observe microbial community changes over time, and enable quick response to events such as blooms.

Persistent presence: The combination of lower cost, scalability, and endurance can greatly enhance data gathering in spatial and temporal domains.

Access to extreme environments: Rapid environmental and microbial change is occurring underneath polar ice, yet direct access by manned vehicles or divers is difficult. Recent discovery of major phytoplankton blooms beneath Arctic ice in the Chukchi Sea (Arrigo et al, 2012) points to our current limited knowledge of these important ecosystems. AUVs have already performed CTD and ice-thickness surveys underneath Arctic and Antarctic sea ice (Wadhams et al, 2006, Williams et al, 2014), and a cytometer payload could enable under-ice microbial surveys.

These benefits could be utilized for many mobile cytometer applications, including the following projects evaluated at the Monterey Bay Aquarium Research Institute (MBARI).

Controlled, Agile, and Novel Observing Network (CANON): This MBARI project aims to monitor microbial populations and their effects on ecosystems in and around Monterey Bay, emphasizing long-term measurements with autonomous platforms, vehicles and instruments (mbari.org, 2015). CANON includes studies of small phytoplankton (*Prochlorococcus, Synechococcus*, and eukaryotes) in offshore mesoscale gyres. Other studies emphasize the development lifecycle of larger cells and algal blooms in the inner bay, including potentially toxic *Pseudo-nitzschia* species. A mobile cytometer would complement physical, chemical sensors, samplers, and ecogenomic instruments such as the Environmental Sample Processor (ESP - see below for more details).

Microbial population changes in ice-covered Arctic waters. Li et al (2009) documented microbial population changes in the Arctic Ocean in concert with climate-driven changes, with picoplankton (less than 2 micrometers) replacing larger cells such as diatoms (2 - 20 micrometers) over a period of several years. These changes may be associated with thinning ice thickness and areal extent as well as freshening and stratification of surface water. Potential shifts in phytoplankton and bacteria community structure will have major repercussions on carbon sequestration at depth in the ocean (Forest et al. 2011). Thus far most sampling had been performed in open water, and much more would be learned from under-ice sampling with a mobile cytometer.

But before autonomous mobile cytometers become reality, we must also consider the technical challenges of operating instruments aboard autonomous vehicles:

Power: Energy is a precious commodity on most autonomous vehicles. Some of today's marine cytometers consume more than 35W when sampling, and must sample for several minutes at least. These power levels could seriously decrease the endurance of small vehicles.

Size: Even compact marine cytometers such as SeaFlow (Swalwell et al, 2011) in its present configuration, are roughly the size of a large microwave oven, and exceed the payload capacity of many vehicles.

Telemetry: Some autonomous vehicles use cell phone networks close to shore but must use low bandwidth, expensive satellite links when far from shore or in remote regions.

Limited telemetry links pose challenges for applications that require near real-time data telemetry to shore.

Environment: The vehicle and its cytometer should be operable within the depth and temperature range of the target phytoplankton, i.e. the photic zone; between 0 and about 200 meters, and from -1.8C to about 38C in the tropics. The instrument should be rugged enough to withstand mechanical shock and vibration during a deployment and recovery cycle, and should be operable within expected tilt/pitch angles during a deployment.

Risk of vehicle loss: Today's cytometers generally cost on order of $100K and more; if the vehicle is lost, then so is an expensive instrument. High cytometer cost also discourages procurement of multiple instruments, e.g. for deployment on a fleet of vehicles.

Table 1 shows characteristic payload volume and power characteristics for just a few autonomous vehicles with endurance of a week or longer; images of the vehicles themselves are shown in Figure 3.

TABLE 1

Some key characteristics of the Liquid Robotics *Wave Glider SV3*, Scripps' *Spray* glider, and MBARI's *Long Range AUV.* Actual endurance will depend on energy needs of integrated instruments, including a cytometer.

	Wave Glider SV3	*Spray* glider	*Long Range AUV*
Type	wave-propelled autonomous surface vehicle	buoyancy-driven glider	propeller-buoyancy hybrid
Approximate payload dimensions (cm)	2 x deck boxes: 380x380x127 1 x deck box10x37x17 towed body: 15 dia x 100 len	11 dia x 15 len	30 dia x 33 len
Max payload weight (kg)	45	3.5	10
Hotel load (watts)	5 - 20	1	50+
Payload energy	150W solar panels, 16 MJ storage batteries	13 MJ (Li primaries)	18 MJ (Li secondaries) 40 MJ (Li primaries)
Typical endurance	months	88 days, 354 profiles	11 days, 400 km 27 days, 1000 km
Reference	liquidr.com, 2015; Brian Kieft (MBARI), personal communication	ucsd.edu, 2015; Brent Jones (MBARI), personal communication	Brian Kieft (MBARI), personal communication

FIGURE 3

Some long-duration autonomous vehicles; (a) *Wave Glider* (b) *Spray* glider (c) *Long Range AUV*. Photographs used with permission (a) Tom O'Reilly (MBARI, 2014); Paul Coenen (MBARI); (b) Kim Fulton-Bennett (MBARI, 2012), Christopher Wahl, (c) Todd Walsh (MBARI, 2013); Brett Hobson, Brian Kieft (MBARI), Thomas Hoover (Ocean Aero)

(a)

(b)

(c)

While these conditions are daunting, we note that increasingly sophisticated instruments are being integrated with autonomous vehicles. MBARI's Environmental Sample Processor (ESP) is an eco-genomic “laboratory in a can”, processing samples with heat and reagents to extract and analyze microbial genetic material (Scholin, 2013). The instrument is made possible by low-power electronics, microfluidic technologies, and stringent power-management strategies. The 3rd generation 3G-ESP is under development for low power mobile platforms, MBARI's Long Range AUV *(LRAUV)* in particular. 3G-ESP is contained in a nose section 28 cm diameter by 61 cm long, draws between 4 and 20 watts, and is rated to 50 meters depth. The prototype underwent successful initial sea trials in Monterey Bay in early 2015. Based on the 3G-ESP and other examples we believe that development of small low-power cytometers for autonomous vehicles is possible in the near future.

<<H2>>Marine Cytometers

At this point only one flow cytometer integrated with an AUV has been reported (Cunningham, 2003). This report describes a flow cytometer integrated with the *AutoSub* AUV, which measured microbial populations across a bloom front in the North Atlantic. *AutoSub* was equipped with a basic science package as well as a water sampler and was deployed into area of interest defined by SeaWIFS satellite ocean color imaging. Cytometric data recorded during this brief hours-long deployment were largely consistent with onboard instruments as well as lab analysis of water samples.

Since 2003, several other cytometers have been developed for autonomous applications aboard ships or stationary platforms such as piers, buoys, and cabled observatories.

The SeaFlow cytometer is designed to automatically acquire data over long periods with minimal human interaction; its primary deployment mode is aboard ships of opportunity where the instrument continually samples water from the ship's intake for weeks at a time (Swalwell et al, 2011). The SeaFlow "virtual core" design eliminates the need for sheath fluid by adding two additional detectors to determine when a cell is optimally positioned for optical measurement. The instrument analyzes cells in the size range of 0.5 - 20 micrometers (pico- and nanoplankton), including *Prochlorococcus* and *Synechococcus*. SeaFlow enables extended two-dimensional surveys of near surface water (Figure 4). Palevsky et al (2013) used continuous underway SeaFlow data to help determine phytoplankton's contribution to the carbon dioxide air-sea flux in Alaskan waters.

FIGURE 4

Distribution of (a) *Prochlorococcus*, (b) *Synechococcus* and (c) nanoplankton in Hawaiian waters, as measured by the SeaFlow cytometer. Large spatial scale views are on the left, finer scale on the right (used with permission, from Swalwell et al, 2011).

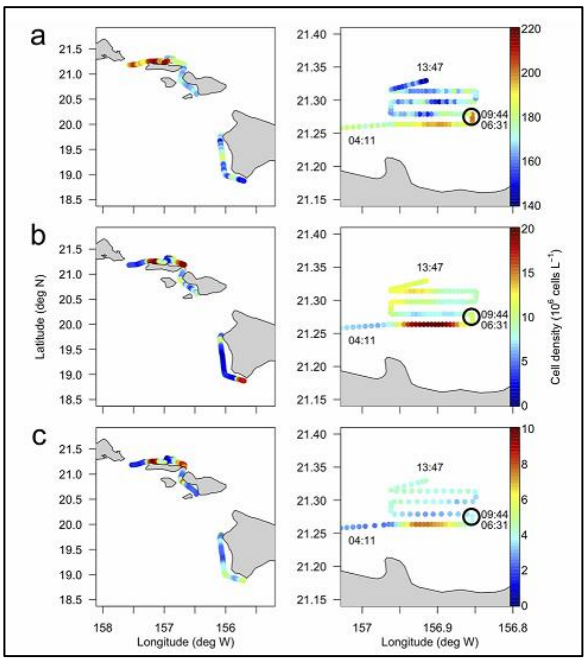
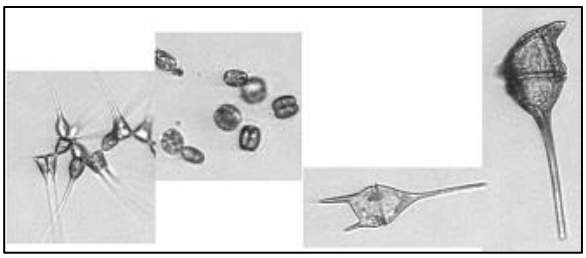


FIGURE 5

Monterey Bay phytoplankton images captured by Imaging Flow Cytobot (instrument loaned to MBARI courtesy of Dr. Heidi Sosik, WHOI)



Imaging Flow Cytobot (IFCB) is designed to automatically image, quantify and identify cells larger than 5 micrometers (Olson and Sosik, 2007). IFCB typically draws in a 5 ml water sample every twenty minutes, and triggers a flash lamp and camera when scatter or fluorescence photomultiplier tube (PMT) signal exceeds a preset threshold. Image scale is typically about 0.3 micrometers per pixel, allowing many cells to be analyzed to the genus or even species level (Figure 5). IFCB has been deployed for up to six months on piers and cabled observatories. Campbell et al (2010) describe detection of toxic algal blooms (*Dinophysis*) with IFCB in a Texas estuary; public health officials quickly responded to the detection by suspending shellfish harvesting until the bloom subsided. IFCB draws considerable electrical power (35W continuously) and generates several gigabytes of data per day; thus the instrument is usually deployed at locations with wired connections to shore providing power and data. IFCB was recently deployed on a moored raft in Salt Pond (Nauset, MA), with no wired shore connection. The raft was equipped with solar panels, a bank of batteries and a gasoline generator that automatically recharged the batteries as required. Communication to shore was via radio link to a shore-based Internet connection (Olson, personal communication).

<<H1>>THE FUTURE

Electronics, lasers, software and fluidics have evolved in the years since the first *AutoSub* cytometer deployments, resulting in smaller, lower-power instruments that will likely be compatible with mobile marine robots in the near future.

Sosik and Olson are currently modifying their Imaging Flow Cytobot for integration with autonomous surface vehicles such as *Wave Glider,* autonomous *JetYak* (whoi.edu, 2014), and *AutoNaut* (AutonautUsv.com, 2015). The instrument will be towed behind the vehicle at a depth of several meters. They anticipate the vehicle will provide sufficient power to allow sampling by the instrument once every 20 minutes. All raw data and images will be stored onboard for post-recovery analysis. Selected images and potentially processed data will be returned to shore over the available telemetry channels in near real-time; 3G/4G cellular modem (0.5-4 Mbps) in coastal areas, Iridium satellite (2400 bps) in more remote regions (Sosik, personal communication).

While the Imaging Flow Cytobot will collect invaluable data from near-surface waters, the instrument is currently too large and consumes too much power for a small, long duration AUVs such as *LRAUV*, capable of surveys throughout the photic zone. New marine

cytometers now under development begin to address these AUV constraints. J. Swalwell of the University of Washington is developing an "immersion flow cytometer" that increases signal-to-noise, simplifies flow cell design, and potentially lowers cost (US Provisional Patent Application Serial No. 62/101,036). The instrument utilizes a low-cost primary ball lens with high index of refraction that is partly immersed in the seawater sample. Analogous to oil or water immersion microscopy, the immersion results in an estimated 60% higher numerical aperture than found in SeaFlow and Influx (BD Biosciences, San Jose CA) cytometers. Chromatic aberration in the ball lens causes laser and fluorescent colors to be dispersed behind the lens, similar to the effects of a prism or grating. Filters and detectors for the wavelengths of interest can be optimally positioned behind the lens, resulting in lower contamination by scattered light at each wavelength and a corresponding signal-to-noise increase. Since the primary lens is immersed directly in the sample, the need for small orifices and sample-delivery tubing may be reduced or eliminated. The instrument utilizes the same "virtual core" technology as the SeaFlow cytometer (Swalwell et al, 2011), eliminating the need for sheath fluid. The cheaper lens, improved signal-to-noise, and simplified fluidic design is expected to result in a smaller (shoebox sized), lower power (roughly 15W) and lower cost instrument (Swalwell, personal communication), with a high potential for deployment on an autonomous vehicle.

Wu et al (2013) describe an imaging approach where a plane of 450 nm laser light (“light sheet”) is used to excite cross-sectional fluorescent images, acquired sequentially, as phytoplankton flow through the light sheet. The instrument generates an integrated cross sectional image of each cell, and is capable of flow rates of at least 1 ml/minute, which is four times the flow rate of Imaging Flow Cytobot. The image quality is such that cells larger than about 5 micrometers can often be taxonomically identified; Orientation of the cells can vary as they pass through the light sheet, and the authors discuss taxonomic identification approaches that take this into account. They intend to miniaturize their prototype such that it is compatible with small autonomous platforms such as moorings and AUVs (R. Chan, personal communication).

Though it does not qualify as a "traditional" cytometer, holographic microscopy is another technique that automatically measures individual cell optical characteristics. Conventional microscope optics is replaced by a laser, pinhole, and CCD detector. The device acquires holograms containing three-dimensional information from particles within the analyzed volume, eliminating the need to physically align the cells in single-file. Cell images

can be reconstructed into multiple image planes. Graham and Smith (2010) describe a submersible holographic microscope with 7.4 micrometer spatial resolution; a commercial version of the instrument has been integrated with MBARI's *Dorado* AUV and is being evaluated for analysis of large phytoplankton and zooplankton (sequoiasci.com, 2015). Bochdansky et al (2013) describe a submersible instrument that acquires several holograms per second; a commercial version of this instrument claims sub-micron spatial resolution (4deep.com, 2015); its physical dimensions of 30 cm length and 10 cm diameter make it a potential candidate for integration with a small autonomous vehicle.

Small low-powered cytometers may someday be capable of the many applications that require cell pre-staining and sorting. Recent advances in microfluidics and manipulation techniques embodied by small ecogenomic sensors such as the AUV-integrated Environmental Sample Processor may point the way in this regard (Scholin, 2013).

Consumer markets of electronics such as mobile phones and computers are rapidly driving the miniaturization and cost-reduction of batteries, processors, cameras, and other sensors. Small, simple low-cost imaging cytometers have been implemented on mobile phones for blood analysis and other medical applications in the developing world (Mudanyali et al, 2012). Perhaps some of these innovations could be explored for marine applications as well, that might dramatically lower instrument cost.

Once a cytometer is integrated with a vehicle, "adaptive sampling" could be used to conserve power and telemetry bandwidth, turning on the instrument only when there is likely something interesting to sample. Zhang et al (2010) describe water sampling of thin phytoplankton layers by MBARI's *Dorado* AUV. The AUV flies a vertical saw-tooth profile through regions that may contain layers, and their algorithm triggers a water sampling bottle when fluorescence and backscatter sensors indicate that the vehicle is moving through the center of a layer. Godin et al (2012) describe an algorithm to track moving patches of phytoplankton. The algorithm adjusts vehicle speed, heading and depth based on the chlorophyll signal from an onboard fluorometer, enabling the vehicle to remain near the centroid of the phytoplankton patch as it drifts with the current. The algorithm could be adapted to power on a cytometer only when the vehicle is in a patch, e.g. to allow the instrument to capture the growth and decline of microbes with time.

Some applications require real-time telemetry of cytometer data to shore, e.g. to issue health warnings in case of a harmful algal bloom. But many cytometers generate gigabytes of raw data each day, and telemetry of this data volume is problematic. In coastal regions the

vehicle may use a 3G/4G cell modem, but beyond 100 km from shore an Iridium satellite link (2400 bps, compressed) may be the only option (Kocak and Clark, 2013), although faster satellite links may be available in the near future. These slow links can result in very long transmission times, high modem energy consumption, high airtime costs, and long surface times for autonomous vehicles. Instead of telemetering the entire dataset, onboard processing could return a brief data summary. In the case of cytometer fluorescence and scatter data, onboard cluster analysis could produce a simple list of detected taxa and their abundance(Aghaeepour et al, 2013). Alternatively Li (1997) describes a “cytometric diversity index" in the form of a few floating point numbers that is calculated based on light scatter and chlorophyll fluorescence, and can be used to estimate the structure of phytoplankton communities in real-time, e.g. to track blooms or biological fronts. In the case of image data, Sosik and Olsen (2007) describe image processing "machine learning" software that classifies individual phytoplankton cells to the genus or species level, based on size, shape, symmetry, texture and other characteristics. Their software currently runs on shore-side machines, but they hope to adapt it to the smaller processors on autonomous vehicles.

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