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Biophotonic Coloration and 3-D Texture in the Flexible Skin of Cephalopods

Roger Hanlon
MARINE BIOLOGICAL LABORATORY THE
7 M B L ST
WOODS HOLE, MA 25431015

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14. ABSTRACT

We studied multiple facets of rapid adaptive coloration in squid and cuttlefish. This sophisticated system can produce dozens of body patterns for camouflage and communication in as little as 200 milliseconds, due to direct neural control of skin chromatophore organs and iridescent cells. Key findings were achieved for four objectives. (1) We examined the pattern of afferent input to the skin, with particular attention to innervation of radial muscles of chromatophores. We pursued the skins system design at a finer scale with 3-D electron microscopy of these multicellular organs of coloration. (2) Dynamic 3-D skin papillae were studied in detail and found to have both CNS and peripheral control; expression of papillae was controlled by one set of nerves, while depression of papillae was controlled by different nerves. Behavioral sensorimotor experiments showed that cuttlefish use visual cues (not tactile ones) to match expression of their skin papillae to surrounding rugosity in the adjacent background for effective camouflage. (3) We studied the relative contribution to skin whiteness by cells containing plates by 3-D segmentation of EM data. We discovered structural coloration phenomena in chromatophores thought previously to be solely pigmentary. (4) We examined the three color classes of chromatophores and found that the pigmented granules have three classes of size and shape. We performed mass spec on material of each color, and obtained the first proteome for this tissue. Surprisingly, the structural coloration protein reflectin was discovered in surrounding sheath cells to produce iridescence. Overall, a great deal of data have been accumulated to better understand the form and function of rapid coloration changes that have evolved to such a sophisticated level in this animal group, and advances in translating this basic science information to practical applica

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Biophotonic coloration and 3-D texture in the flexible skin of cephalopods

Principal Investigator: Roger T. Hanlon, PhD

Co-Investigators: Trevor Wardill, PhD, Paloma Gonzalez-Bellido, PhD

Marine Biological Laboratory, Woods Hole, MA 02543 rhanlon@mbl.edu
508 274 3633 (mobile)

ABSTRACT

We studied multiple facets of rapid adaptive coloration in squid and cuttlefish. This sophisticated system can produce dozens of body patterns for camouflage and communication in as little as 200 milliseconds, due to direct neural control of skin chromatophore organs and iridescent cells. Key findings were achieved for four objectives. (1) We examined the pattern of afferent input to the skin, with particular attention to innervation of radial muscles of chromatophores. We pursued the skin's system design at a finer scale with 3-D electron microscopy of these multicellular organs of coloration. (2) Dynamic 3-D skin papillae were studied in detail and found to have both CNS and peripheral control; expression of papillae was controlled by one set of nerves, while depression of papillae was controlled by different nerves. Behavioral sensorimotor experiments showed that cuttlefish use visual cues (not tactile ones) to match expression of their skin papillae to surrounding rugosity in the adjacent background for effective camouflage. (3) We studied the relative contribution to skin whiteness by cells containing plates by 3-D segmentation of EM data. We discovered structural coloration phenomena in chromatophores thought previously to be solely pigmentary. (4) We examined the three color classes of chromatophores and found that the pigmented granules have three classes of size and shape. We performed mass spec on material of each color, and obtained the first proteome for this tissue. Surprisingly, the structural coloration protein reflectin was discovered in surrounding sheath cells to produce iridescence. Overall, a great deal of data have been accumulated to better understand the form and function of rapid coloration changes that have evolved to such a sophisticated level in this animal group, and advances in translating this basic science information to practical applications have been demonstrated.

Objective 1: Design principles for flexible skin.

We delved into the anatomical details of iridophore and chromatophore organization in living squid skin by refining our novel methods of using a variety of vital dyes to bulk-label elements throughout the skin, focusing on the chromatophore system (chromatocyte, sheath cells, afferent nerves, radial muscles, and the flexible collagen-rich layers in which they are embedded), see Fig. 1A for examples. We recently extended vital staining to live hatchling animals where (being

small) the entire pathway can be followed in fluorescence using confocal microscopy from a chromatophore through the entire flexing skin, back through the stellate ganglion in the periphery, and thence to fibers originating in lobes of the Central Nervous System (CNS, including posterior chromatophore lobes and more anterior lobes).

We were able to extend our study of skin microanatomy to the EM level in very high resolution 3-D (<= 50 nm isotropic) by additional serial block-face imaging first applied to this system in the forerunner to this grant (Fig. 1B). These have shown unexpected complexity of the skin at the nanoscale including matrix with fibroblasts and collagen, the radial muscle structure (including direct muscle-muscle contacts that appear functionally specialized) and the bundles of axons innervating them, which are more precisely arranged and more numerous than expected.

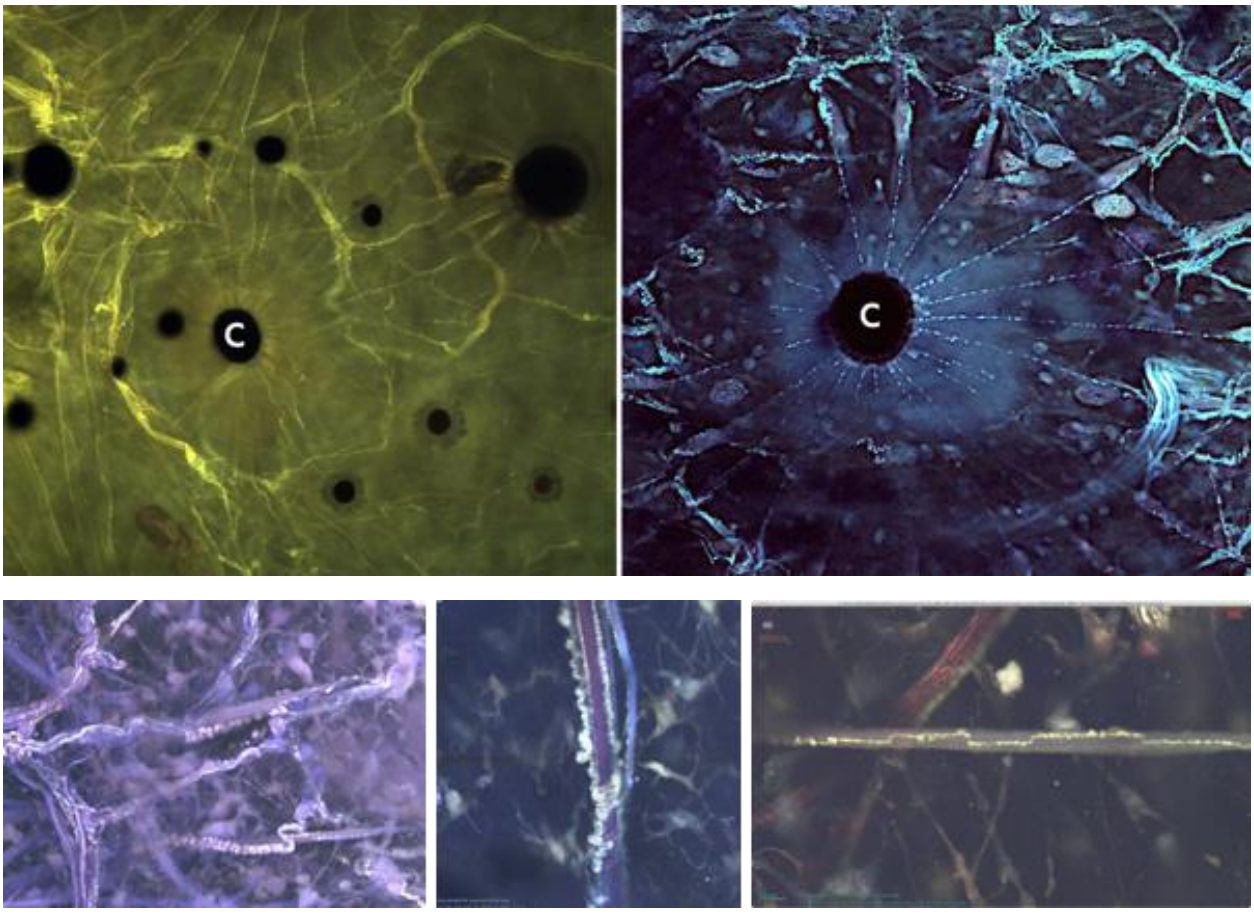

Fig. 1A. Nerve bundles and single axons targeting chromatophore muscles, stained with vital dyes. C is the chromatocyte that contains pigment granules. (Scale: FOV ~ 1mm across in top row; bottom left FOV is ~500 um across, middle FOV ~250 um across; right: FOV ~225 um across).

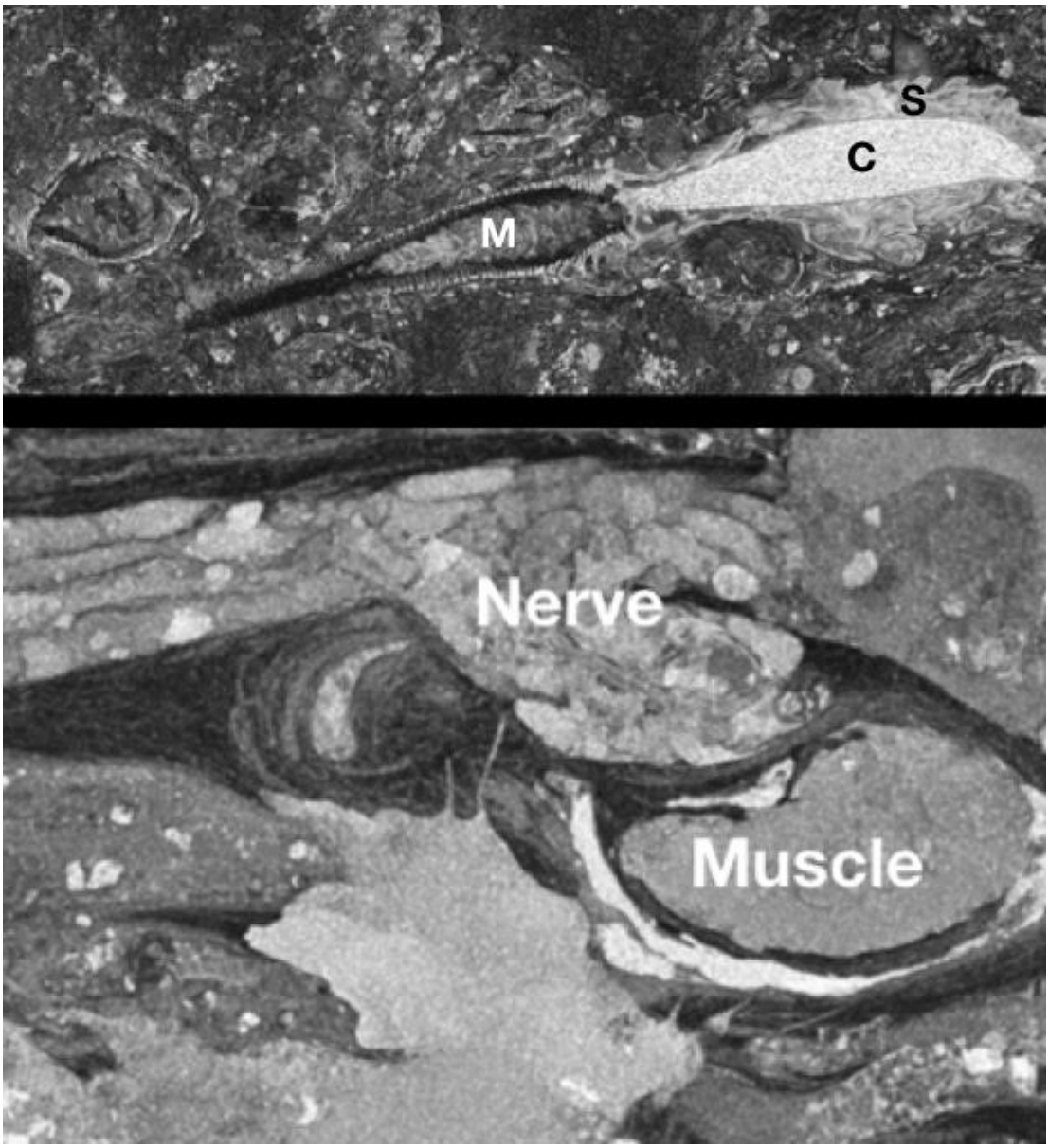

Fig. 1B. Top. Renderings of 3-D serial-block-face electron micrographs showing chromatophore (C) with sheath cells (S) and attached radial muscle (M). Bottom. Closer view of multi-axon nerve bundle branching and approaching a radial muscle. (Scale: top: FOV is ~5 um wide; bottom: muscle profile is ~10 um across,~25 um FOV.)

These anatomical findings in both living and fixed issue have allowed us to better appreciate the numerical scale and organizational complexity of the innervation of this intricate brain-controlled system that has evolved for expressing and suppressing coloration. It may be that the local axonal complexity in the periphery confers on this system the property of local dynamic pattern generation, so that the CNS need only send detailed modulatory information but may not have to specify peripheral behavior at the level of every muscle twitch.

Objective 2: Shape-changing 3D skin: morphology and neural control of papillae.

We determined that the coordination of papillae expression in the skin of cuttlefish with background rugosity is controlled by vision, not by tactile feedback. That is, a cuttlefish views the surrounding substrate and – with monocular vision laterally (their eyes are apposed) – determines the fine 3-D structure of surrounding background and then expresses their skin papillae to match that surrounding 3-D physical texture. It is roughly analogous to a dynamic gilly suit. This was published by Panetta, Buresch & Hanlon (2017).

We have created a full inventory of the many types of papillae in two cuttlefish species: the common European cuttlefish *Sepia officinalis* and the giant Australian cuttlefish *Sepia apama*. In both species we have identified camouflaging behaviors for which 3-D papillae expression are essential for many grades of concealment. Figure 2 shows the details that we were able to extract from extensive analysis of underwater videos and photographs as well as laboratory experiments. It is important to realize that each type of named papilla is a separately controlled entity. That is, for *Sepia apama*, there are 13 types of papillae, and each can be expressed alone or in combinations with other papillae types. Thus, the neural control of papillae is precise and complex, suggesting the key importance of 3-D physical texture for effective camouflage.

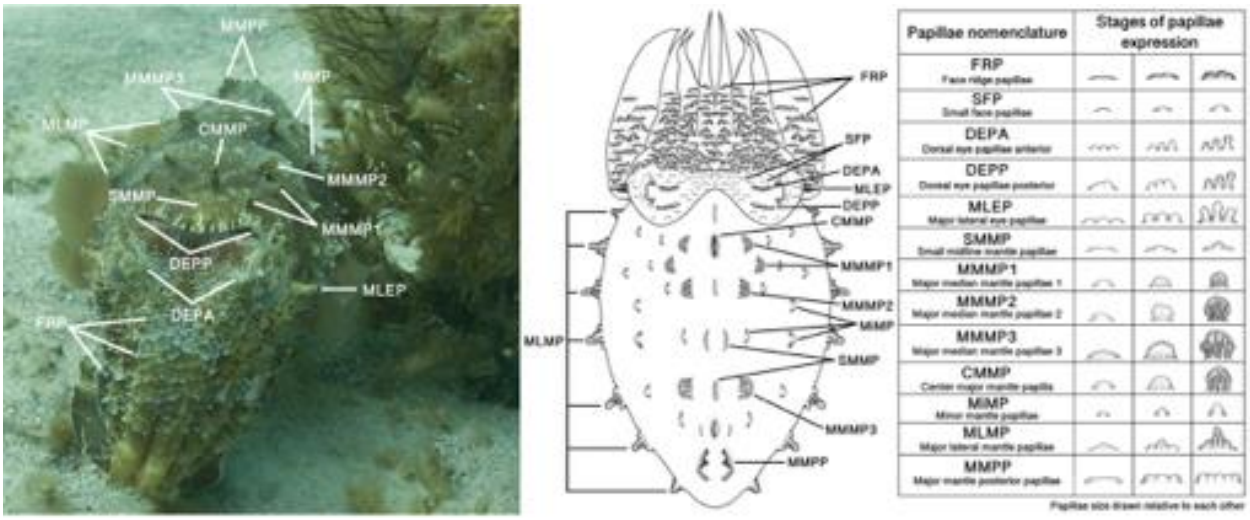

Fig. 2. Thirteen papilla types in *Sepia apama*. The cuttlefish is ca. 500g.

Neural control of skin papillae has not been studied previously. We determined by selective denervation, and by backfilling of cell bodies in the stellate ganglion, which regions govern expression of many of these papillae. We found where and how to electrically and chemically stimulate to raise and to lower some papillae. Surprisingly, we found that there are dedicated nerves to raise papillae to any level of expression, but different nerves to activate musculature to lower papillae. Details of our findings have been published by Gonzalez-Bellido, Scaros, Hanlon, Wardill (2018) and the essence is illustrated in Fig. 3.

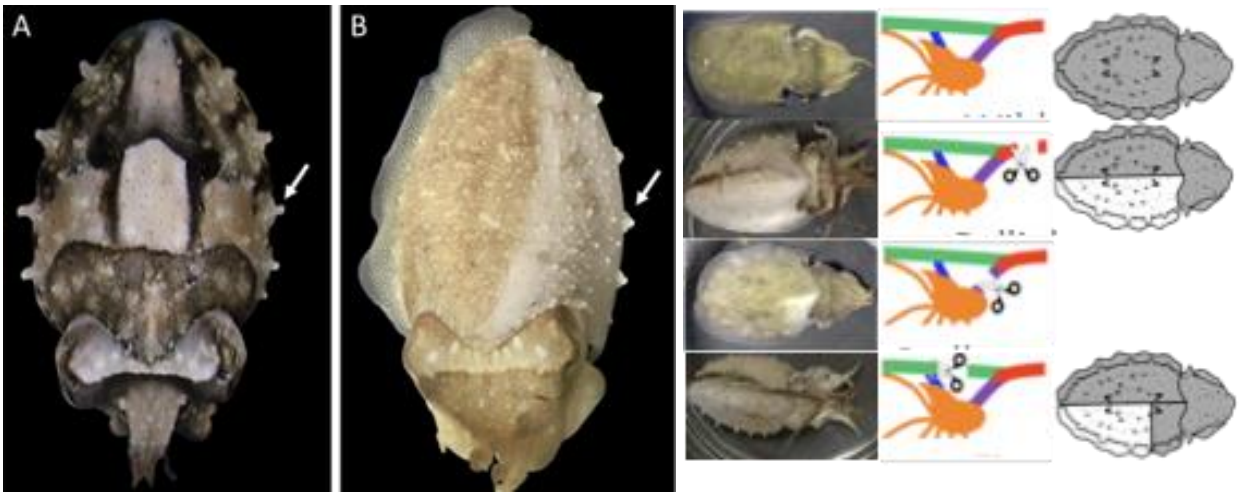

Fig. 3. Specific nerve transections differentiate control of papillae from chromatophores.

The other question we began to address was how energetically demanding the papillae might be since they have to be held in different grades of expression for long periods for camouflage on different backgrounds. We found evidence that the papilla muscle system has catch-like properties. Details are published by Gonzalez-Bellido, Scaros, Hanlon and Wardill (2018).

Papillae receive innervation that employs multiple transmitter types in axons of varying caliber. With phalloidin (to show muscles) and immunocytochemistry targeting glutamate, ChAT, 5HT, FMRF and phosphorylated neurofilaments (to reveal mature axons) we were able to follow fibers onto spatially and structurally distinct subsets of muscles within *Sepia* papillae, exemplified in Fig. 4.

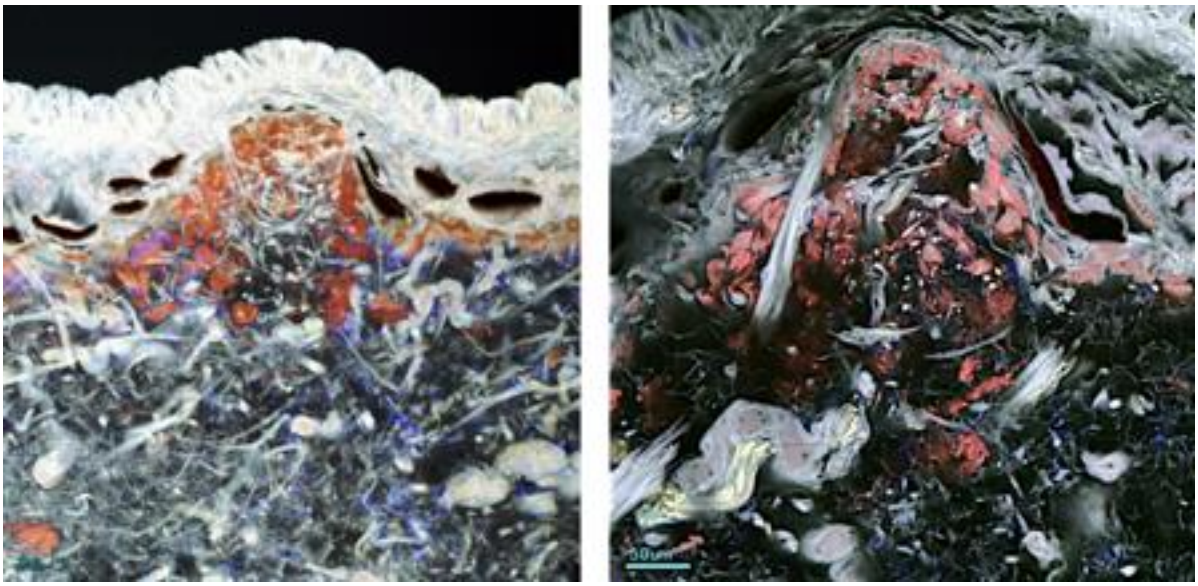

Fig. 4. Cross sections of immune-stained papillae imaged with confocal microscopy (scale bar 50 microns).

To translate our basic research to applied materials science and engineering, we collaborated with Cornell University (laboratory of Robert Shepherd in Department of Materials Science and Engineering) to extract some of the biological principles that we discovered to generate the first synthetic material able to deform similarly to papillae (Fig. 5). We published this in Science (Pikul et al., 2017). This may inspire new classes of soft actuators that are thought to have wide applications once the materials and control are refined.

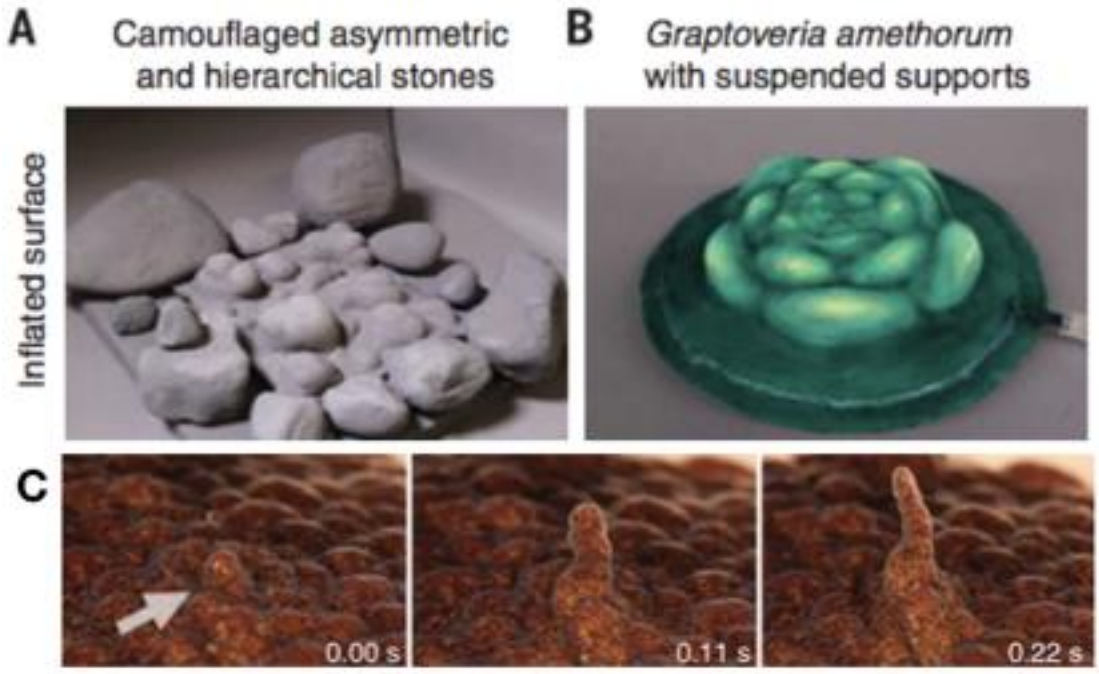

Fig. 5. Bio-inspired engineering of soft actuators. A. A 22- by 22-cm membrane programmed to inflate into nonsymmetric and hierarchical stone shapes. Natural river stones with the same color encircle the membrane. (B) A membrane programmed to inflate into the shape of a G. amethorum plant. The leaves are arranged in a spiral around a center point and use suspended mesh supports to maintain the high-aspect-ratio mesh patterns. C. A conical papilla (~4 mm high) in *Octopus rubescens* that dynamically extends or retracts in ~220 ms.

By serendipity, we were conducting diving research in the Caribbean for part of another camouflage grant and we discovered a small fish that had fast changeable camouflage as well as physical structures that looked at first like the dynamic papillae of cephalopods. These turned out to be 3-D dermal flaps – not extendable bumps – with a very different mechanical actuator that does not provide the degrees of freedom of expression that cuttlefish have. They are essentially fully "off or on." Nevertheless, they may provide some useful inspiration to DoD for less demanding aspects of concealment. This was published by Allen, Akkaynak, Sugden and Hanlon (2015).

Objective 3: Biophotonic skin structures: ultrastructure, spectrometry, biochemistry and modeling of pigments and structural coloration elements.

We have evaluated for distinctive regions of the body of the cuttlefish the relative contribution to the appearance of whiteness by leucocytes (cells with Mie-scattering spheres (containing

reflectin) and iridocytes (containing similar material in the shape of plates arranged as Bragg stacks, that are known to generate structural iridescence (published by Hanlon, Mathger, Bell, Kuzirian, Senft, 2018).

With assistance from Drs. T. Szasz and Berali Runesha at the University of Chicago Research Computing Center, we have been attempting to obtain 3-D segmented morphometrics from 3-D EM data to quantify the numbers, packing and orientation of plates found in iridocytes of the *Sepia* fin spot (Fig. 6). We expect that information to help us to evaluate the optical contribution of cells packed with these unusual inclusions and to help us answer the question "why are platelet-filled cells, that elsewhere generate structural color, nevertheless present in skin regions that exhibit bright whiteness?" This has been a central objective of the entire grant and has proved to be so formidable that we do yet know definitively what the answer is; we are close though since in March 2019 we may have found a way to segment the wavy platelets.

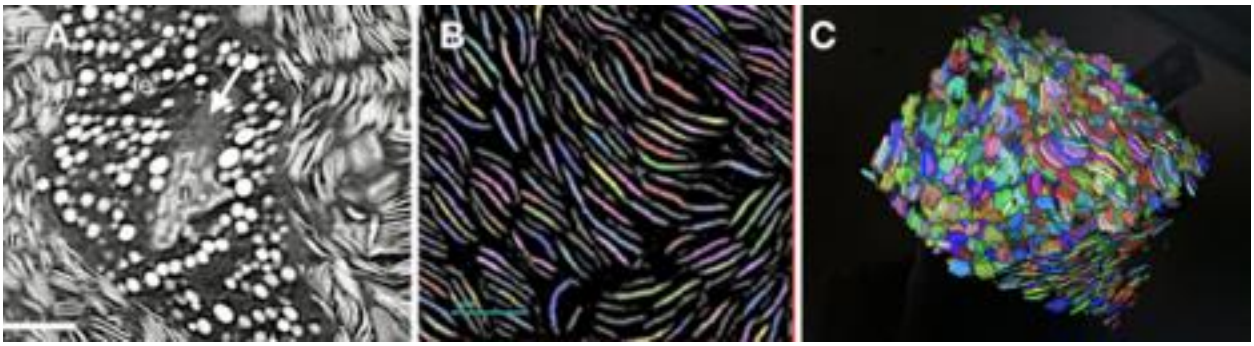

Fig 6. Bright whiteness from leucophores in cuttlefish. A. a cell from a *Sepia* fin spot that we previously determined generated Mie scattering. B. 2-D cross section of plates from an adjacent cell, segmented in 3-D by Dr. Szasz. C. A 3-D rendering of a portion of a cell containing many small platelets. (Scales: [A] bar is ~2 um, [B] image full width is ~10 um; [C] cube is ~10 um on a side.)

We discovered that under the proper lighting conditions even chromatophores can exhibit vibrant iridescent color, evidently by structural means (see Figs. 7, 8; published in Williams et al, 2019).

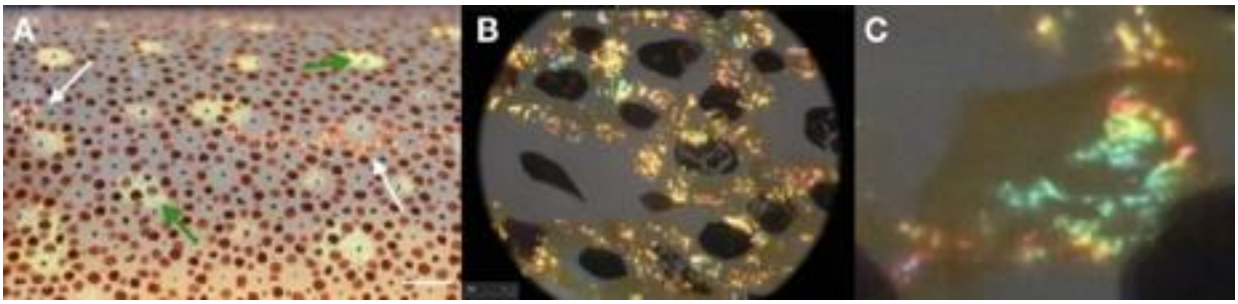

Fig. 7: A. Low power view of living squid mantle. Bright reflection (right white arrow) is from open yellow chromatophores. This structural color is distinct from that produced by iridophores (green arrows). B and C show the intense iridescent quality of the chromatophore reflection.

(Scales: A. bar is 3 mm.; B expanded yellow chromatophores are ~450 um in diameter; C full width FOV is ~500 um wide)

With colleagues at Northeastern University who are expert in Mass Spectrometry, we obtained the first proteome of the chromatophore. *This revealed the unexpected finding that reflectin is present in the chromatophore organs*. Using immunocytochemistry (based on reflectin antibodies kindly donated by colleagues Dr. Wendy Goodsen of AFRL and Dr. Dan Morse of UCSB) we found reflectin associated with the sheath cell component of the organ. The geometry underling this structural coloration remains a mystery, since with EM no plates are seen in the vicinity. This is an intriguing finding and opens the door to investigate novel ways that structural coloration can be achieved with reflectin that is not aggregated into plates or spheres.

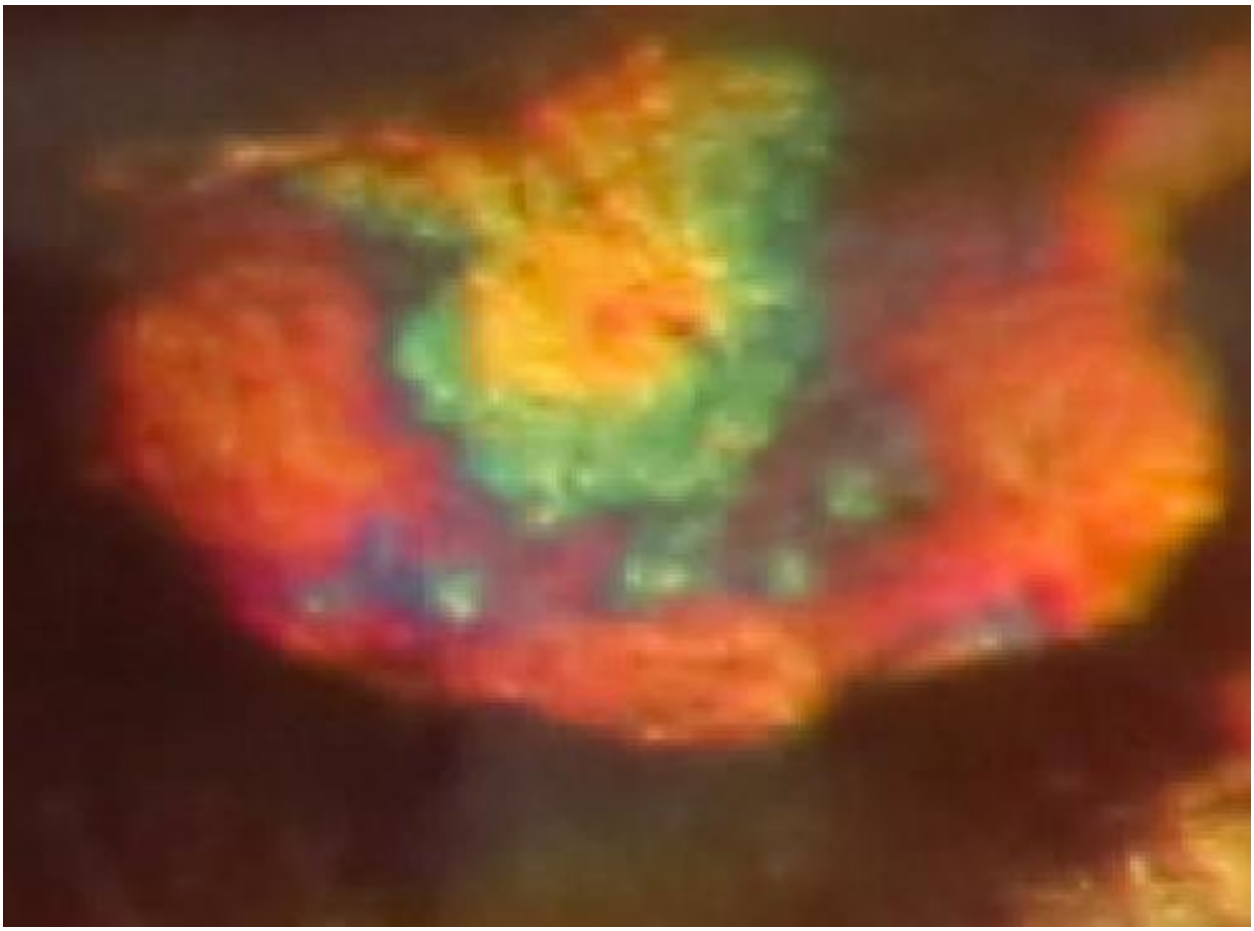

Fig. 8. Close up of a single expanded yellow chromatophore, exhibiting a wide range of reflected color, possibly correlating with chromatocyte and sheath cell topography. (Scale: full image FOV is ~500 um wide)

Cephalopod visual systems are well known for being able to detect polarized light. While this newly discovered chromatophore phenomenon also has a polarization component, we additionally earlier undertook a study of the polarization signal from squid skin in greater detail, focusing in that work on the classical source of cephalopod structural coloration: iridophore

patches in squid mantle (manuscript in preparation; Temple et al.).

Objective 4: Mechanisms of colorant transposition that maintain uniform appearance during differential expansion of pigmented chromatophores.

By enzymatically dissociating chromatophores from squid mantle we found that the saccules of each color contain granules of characteristic size and shape (Fig. 9).

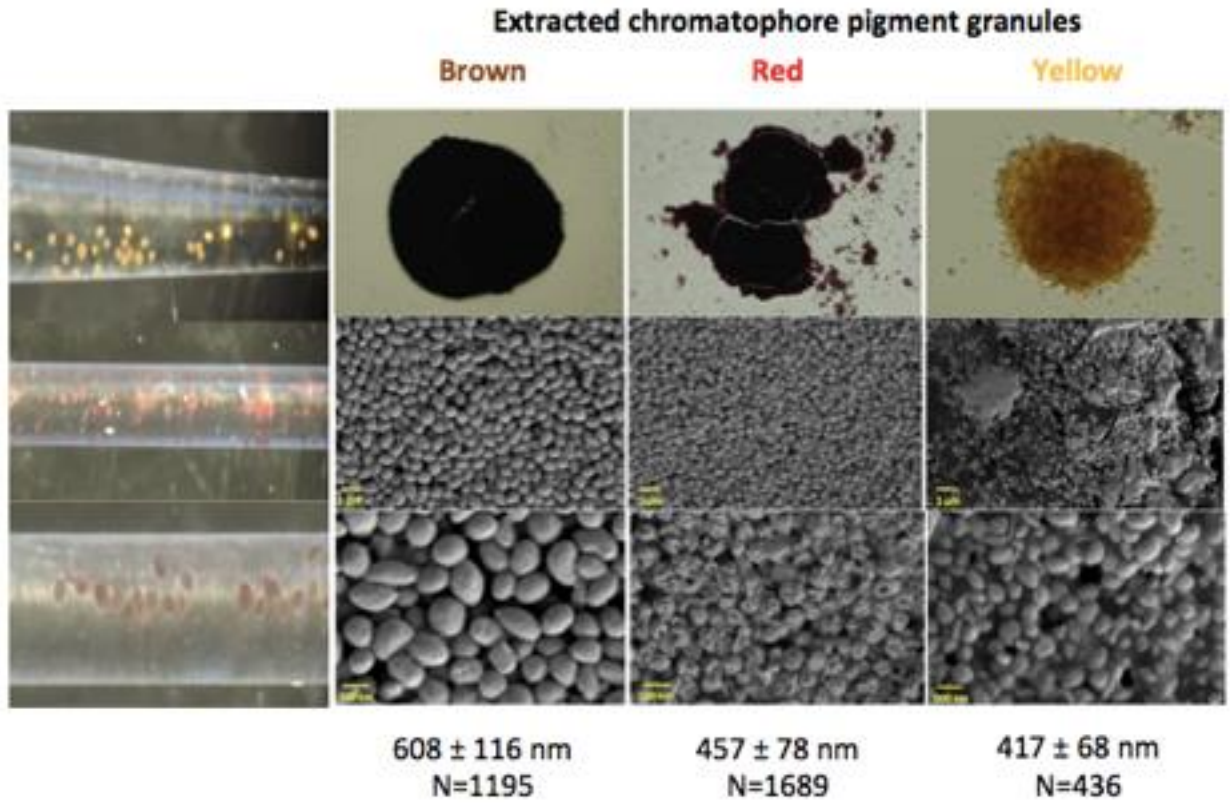

Fig. 9. At left at low magnification in pipettes are chromatophore saccules (ca. 500 um diameter) manually harvested by color: yellow, red, brown. At top right are individual saccules by light microscopy showing transmitted color. Below are SEM images showing the relative sizes (in nm) and shapes of the pigmented granules of each color class.

We have long been interested in how the granules maintain neighborhood spacing as the chromatophores actuate. We obtained additional information about this from 3-D EM data. We determined that the interior of the chromatophore saccule is better interpreted as a dense spongium rather than a system of tethered spheres (Figs. 10, 11).

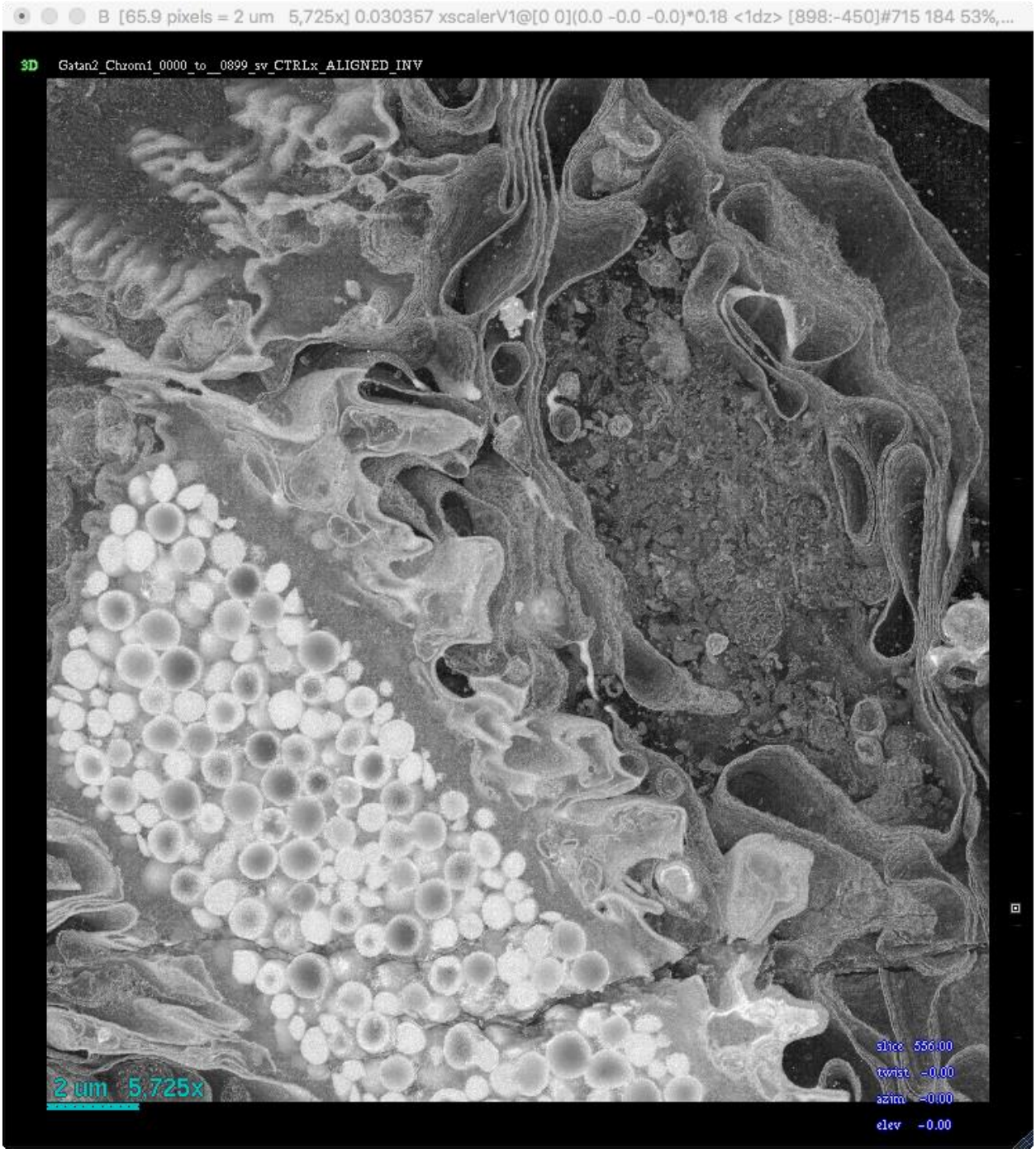

Fig. 10: Rendering of a 3D serial block face EM image stack. At lower left is a chromatophore saccule, filled with (red/brown) pigmented granules immersed in a fine-grained matrix. Fingers of chromatocyte membrane interdigitate with several layers of sheath cells (one filled with cytoplasmic contents) extending to upper right. At upper left is a radial muscle, showing complex membranous ribbing. (Scale bar 2 um.)

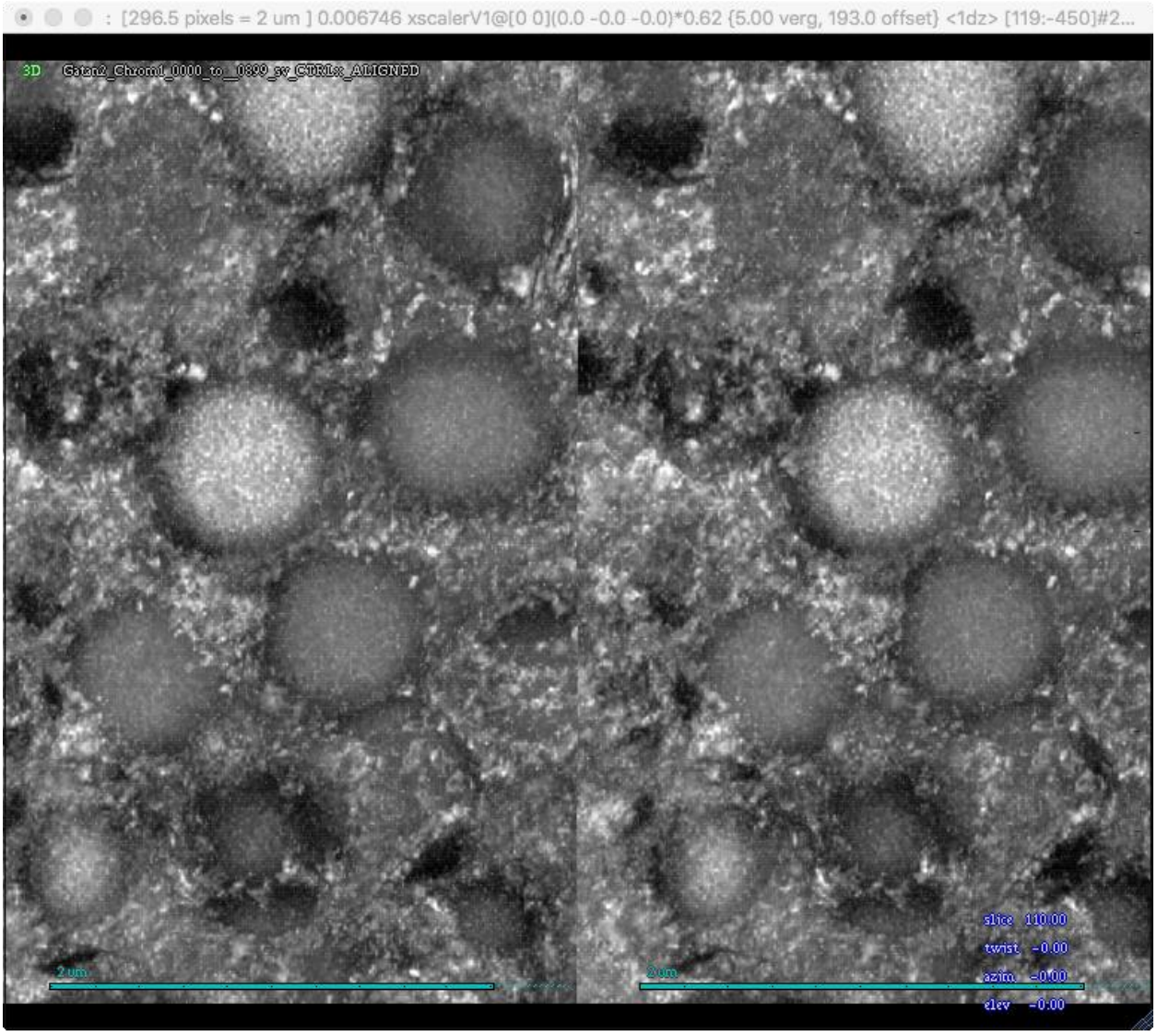

Fig. 11. Close up rendering of granules within the saccule (stereo pair). A dense flocculant material composed of particles fills the interstices between granules. We posit that this material is flexible and remains cohesive enough to prevent individual granules from moving long distances during actuation. (Scale bar 2 um.)

We obtained mass spec information concerning the differential biochemical makeup of the three colors of chromatophore. For details, see Williams et al. (2019). In addition to reflectin, found to be associated with sheath cell, crystallin proteins were found in the chromatophores. We now hypothesize that crystallin (potentially as part of the fine grain visible within each granule in Fig. 11) may play a role in pigment stabilization and may help to maintain color saturation.

Summary of discoveries.

Cephalopod chromatophores. These were thought to be exclusively pigmentary but we discovered serendipitously that they can produce structural iridescent color from reflectin proteins. This unprecedented tight co-localization of pigmentary and structural coloration in the same dynamic organ is unique in the animal kingdom and lends fresh perspective on how diverse and dynamic changeable materials might be developed by materials scientists and engineers.

Neural control. Iridescence and skin papillae (for 3D skin texture for camouflage) neural control takes place partly in the peripheral nervous system, unlike the chromatophores, which have all neural cell bodies in the CNS.

Papillae control and emulation. Papillae expression is guided by vision and there are many shapes and types of papillae, each controlled separately by the brain. Our 2017 paper in *Science* shows how new classes of soft actuators might now be produced by materials scientists, all based on our biology of the muscular hydrostatic papillae in cuttlefish.

Ultrawhite structural coloration. The whitest-white known from the animal kingdom occurs in the leucophores of cuttlefish (but also in beetles) and we have shown new data on the morphology of those leucophore cells that contain reflectin proteins.

COLLABORATORS

Dr. Leila Deravi at Northeastern has been a close collaborator during the past 3 years as we study chromatophores; in our 2019 paper in Nature Communications, our lab personnel shared first authorship and she and I shared Corresponding Authorship. We also collaborated with Dr. Rob Shepherd and his lab at Cornell for the 2017 Science paper. For 2 years we have been collaborating with postdoc Dora Szasz and Dr. Hakizumwami Birali Runesha, Research Computing University of Chicago, on segmentation of iridophore platelets in white fin spots (this is an ongoing aspect of this grant). Drs. Richard Baraniuk and Naomi Halas of Rice University have devised a unique lensless camera inspired by our work on cephalopod skin; they and postdocs were in the Hanlon lab at MBL during February 2019 testing a related camera system that can provide spectrometry of cephalopod skin in the intact animal. Dr. Evelyn Hu of Harvard has been working with us for 2 years to study the external shape and internal structure of chromatophore pigment granules (see Fig. 9 this report). Eventually there will be a publication emerging from this study. In the first two years of this grant we worked with Dr. George Kattawar of Texas A & M for modeling of spheres and plates that produce ultra white reflection from leucophores.

INVITED SEMINARS

In the last year of this grant (2018), R. Hanlon gave more than a dozen talks that involved work presented in the report. Examples are MIT Lincoln Labs, Yale Dept of Cellular & Molecular Physiology, PASPCR Melanoma meeting in Oregon, Global Frontiers in Science and Technology at Harvard, the Brains Minds & Intelligence class in Woods Hole, Logan Science

Journalism Fellows, Bristol UK Camouflage Meeting, U Wisconsin Color Symposium.

LITERATURE PRODUCED (in descending chronicle order)

Williams, TL, Senft SL, Yeo, J, Martin-Martinez, FJ, Kuzirian AM, Martin CA, DiBona CW, Chen, C-T, Dineen SR, Nguyen HT, Rosenthal, JJC, MacManes MD, Buehler MJ, Hanlon, RT, Deravi, LF. (2019) **Dynamic pigmentary and structural coloration within a cephalopod chromatophore organs**. *Nature Communications* 10: 1004.

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Gonzalez-Bellido, P. T., Scaros, A. T., Hanlon, R. T., & Wardill, T. J. (2018). **Neural control of dynamic 3-dimensional skin papillae for cuttlefish camouflage**. *iScience*. doi:10.1016/j.isci.2018.01.001

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Panetta, D., Solomon, M., Buresch, K. and Hanlon, R.T. (2017) **Small-scale rearing of cuttlefish (*Sepia officinalis*) for research purposes**. *Marine and Freshwater Behaviour and Physiology* 50 (2): 115-124.

Panetta, D., Buresch, K., & Hanlon, R. T. (2017). **Dynamic masquerade with morphing three-dimensional skin in cuttlefish**. *Biology Letters,* 13, 20170070.

PLANNED PUBLICATIONS FROM THIS GRANT

Temple SE, Gonzalez-Bellido PT, York T, Gruev V, Roberts N, Hanlon RT, Wardill TJ.
Squid dynamic iridescence provides polarized signals detectable by conspecifics.

Senft SL, Kuzirian AM, Hanlon RT.
Ultrastructural muscle specializations revealed by 3-D EM may augment nerve-driven waves of cromatophore patterning.

Hanlon, RT and O Cattau
Ultrafast physiological color pattern change in cephalopods