Evaluation of fluorescence-based viability stains in scleractinian coral cell cultures

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Abstract

The application of established cell viability assays such as the commonly used trypan blue staining method to coral cells is not straightforward due to different culture parameters and different cellular features specific to mammalian cells compared to marine invertebrates. Using Pocillopora damicornis as a model, we characterized the autofluorescence and tested different fluorescent dye pair combinations to identify alternative viability indicators. The cytotoxicity of different representative molecules, namely small organic molecule, protein and nanoparticles (NP), was measured after 24 hours of exposure using the fluorescent dye pair Hoechst 33342 and SYTOX® orange. Our results show that this dye pair can be distinctly measured in the presence of fluorescent proteins plus chlorophyll. P. damicornis cells exposed for 24 hours to Triton-X100, insulin or titanium dioxide (TiO2) NPs, respectively, at concentrations ranging from 0.5-100 µg/mL, revealed a LC50 of 0.5 µg/mL for Triton-X100, 20 µg/mL for TiO2 NPs and an average 20% reduction in viability at 100 µg/mL for insulin. The workflow presented here provides a general framework for customizing dye pairs for cell viability assays considering the species- and genotype-specific autofluorescence of scleractinian corals.

Introduction

Cytotoxicity, the potential to cause cell death, is measured via different endpoints[1]. These endpoints can include membrane integrity, mitochondrial function, proliferation, and apoptosis *versus* necrosis. It is important to systematically combine multiple endpoints, such as membrane integrity and cell death mechanism, to gain insight into the potential pathways involved in cellular toxicity. The most common way to measure these endpoints is by using colorimetric or fluorometric assays which involve the addition of an indicator dye. Many indicator dyes have been developed for different endpoints and finding the right type of dye hinges on the cell model system studied, desired throughput, instruments available for analysis, and cost of reagents[2]. Fluorescent indicator dyes (fluorophores) are amongst the most common live/dead stains used in vertebrate cell culture since many instruments (microscopy, spectroscopy, flow cytometry) can detect fluorescence and few vertebrate cells possess endogenous fluorescent proteins. Fluorophores used to assess cytotoxicity mostly emit visible light in the blue, green and red color bands. Table 1 includes common fluorophores used to assess membrane integrity and distinguish between live and dead cells when conducting viability assays.

Table 1
Fluorophores tested on coral cells and conclusions

Fluorophore λ_{ex} / λ_{em} (nm)	FC*	Type	Findings
Hoechst 33258, 352/461 (blue)	40 µM	Membrane permeable, non-toxic	Does not penetrate the cells well
Hoechst 33342, 361/497 (blue)	40 µM	Membrane permeable, non-toxic	Bright stain but needs a minimum of 30 min incubation at 25°C
NucBlue, 360/460 (blue)	NA**	Membrane permeable, non-toxic	Bright stain, Hoechst 33342 is active agent here
Calcein AM blue, 408/450 (blue)	2 µM	Membrane permeable, non-toxic	Stains but weakly fluorescent
Calcein AM, 496/515 (green)	2 µM	Membrane permeant	Overlaps with GFP, cannot be deconvoluted
Ethidium homodimer, 528/617 (red)	4 µM	Membrane impermeant	Efficient and visible staining but must be manually deconvoluted from chlorophyll spectrum
POPO™-3 iodide, 534/570 (orange)	2 µM	Membrane impermeant, non-toxic	Cannot be deconvoluted using the RFP filter cube but laser scanning microscopy will work
SYTOX™ Red, 540/658 (red)	0.2 µM	Membrane impermeant, non-toxic	Efficient and visible staining but must be manually deconvoluted from chlorophyll spectrum
SYTOX™ Orange, 547/570 (orange)	0.2 µM	Membrane impermeant, non-toxic	Efficient and visible staining but can stain contamination if present
BOBO™-3 iodide, 570/602 (orange)	2 µM	Membrane impermeant, non-toxic	Cannot be deconvoluted using the RFP filter cube but laser scanning microscopy will work
Propidium iodide, 585/617 (red)	1 µg/mL	Membrane impermeant, toxic ≥ 24h	Efficient and visible staining but must be manually deconvoluted from chlorophyll spectrum
*FC: final concentration in cell suspension			
** Not specified by vendor			

The need for cellular-scale understanding of mechanisms such as symbiosis, calcification, wound healing and bleaching is increasing as reef-building corals (Scleractinia) are more than ever under stress

from rising sea surface temperatures, ocean acidification, diseases, pollution and habitat loss[3]. While many studies have focused on colony-scale or polyp-scale dynamics, cellular studies are less common because the scientific community has only reported generating axenic, immortal scleractinian coral cell cultures twice since the early 1990s (see[4,5]) and concerns remain regarding contamination and cell identification as our knowledge of the coral holobiont grows[3,6]. The further development of scleractinian coral cell cultures requires quantitative assessment of survivorship through basic live and dead cell counts. Survivorship being critical to determining culture method success, it is important to have a clear approach for quantifying population viability without overlap between indicator dyes and endogenous fluorescence (autofluorescent signals measured in different coral species Fig. 1–2). The use of trypan blue, a colorimetric, cell impermeant stain, to quantify cell death is the most reported method in relation to coral cell cultures despite its limitations. The main issue with trypan blue is its capacity to bind to proteins in the cell suspension, not just that released by dead cells. This results in blue aggregates that hinder the cell count. There is evidence that trypan blue can be toxic in the case of longer incubation times[7]. Furthermore, ideally, dyes should be used in pairs with one dedicated to live cells and the other to dead cells for increased accuracy. Calicoblastic cells, i.e. calcifying cells, are ~ 10x smaller than symbiotic gastrodermal cells and nematocysts (also referred to as cnidocytes, stinging cells) Fig. 2. Without a dye pair combination for viability, cell density can easily be underestimated and smaller cells can be inadvertently disregarded as debris.

Fluorescent proteins extracted from marine invertebrates, especially Cnidarians, have helped advance biological research thanks to their capacity to serve as molecular markers and biosensors[8]. The green fluorescent protein (GFP) was first characterized in the jellyfish *Aurelia victoria* (*Aequorea victoria*)[9] and has now become one of the most useful tools in modern science and medicine[10]. Multiple coral chromophores have been identified so far including cyan fluorescent protein ($\boldsymbol{\lambda}_{ex}$ 404–477 nm, $\boldsymbol{\lambda}_{em}$ 485–495 nm), green fluorescent protein ($\boldsymbol{\lambda}_{ex}$ 478–508 nm, $\boldsymbol{\lambda}_{em}$ 500–520 nm) and red fluorescent protein or symbiotic chlorophyll ($\boldsymbol{\lambda}_{ex}$ 560–589 nm, $\boldsymbol{\lambda}_{em}$ 576–595 nm), and non-fluorescent pink-purple-blue ($\boldsymbol{\lambda}_{ex}$ 560–588 nm)[11–13] (Fig. 1). Yellow fluorescent proteins ($\boldsymbol{\lambda}_{ex}$ 425–550 nm, $\boldsymbol{\lambda}_{em}$ 525–570 nm) are also present in corals but appear to be specific to *Agaricia* sp.[14]. The use of fluorophores in cells with endogenous fluorescent proteins, such as scleractinian coral cells, presents a challenge because fluorophore emission wavelengths can overlap with that of the endogenous fluorescence emitted by fluorescent proteins or symbiotic algae cells (chlorophyll) hosted within coral cells, thereby confounding the signal.

The objective of this study is to tailor cell viability and cytotoxicity assessments *via* membrane integrity to scleractinian coral research by taking into consideration the endogenous fluorescent signal. To this end we tested established membrane integrity assays developed for vertebrate cells on *Pocillopora damicornis* (green phenotype, Fig. 2) cell cultures and investigated the overlap between assay fluorophores and endogenous proteins. We then used the resulting protocol to test the cytotoxicity of three representative classes of molecules, namely the small molecule Triton X-100, the protein insulin,

and a representative engineered nanoparticle, titanium dioxide, as a proof-of-concept and first assessment of the effects of different types of molecules on coral cell physiology.

Results

Tissue of scleractinian coral *Pocillopora damicornis* (Fig. 2) was dissociated from the skeleton using calcium and magnesium-free seawater incubation for 1 hour as described in our earlier publication[3]. The mixture of cells was washed and resuspended in complete culture medium (CM, see **Material & Methods** for details). The resulting cell suspensions were then observed under a confocal laser scanning microscope and analyzed using a fluorimeter to measure the autofluorescence of *P. damicornis*. The excitation-emission matrix (EEM, Fig. 3) shows five emission peaks representing tryptophan (λ_{ex} 280 nm, λ_{em} 350 nm, **A**), cyan fluorescent protein (**B**), green fluorescent protein (**C**), red fluorescent protein (**D**) and the endosymbionts' chlorophyll (**E**). The lambda scans confirmed these fluorescent signals (**Supplementary Materials Fig. S1 A-G**).

The fluorophores tested on the *P. damicornis* cells were Hoechst 33258 (blue, all cells), Hoechst 33342 (blue, all cells), NucBlue (blue, all cells), Calcein blue AM (blue, live), Calcein AM (green, live), Ethidium homodimer (red, dead), POPO™-3 iodide (orange, dead), SYTOX® Red (red, dead), SYTOX® Orange (orange, dead), BOBO™-3 iodide (orange-red, dead), and Propidium iodide (red, dead). The choice of fluorophores to test was guided by emission spectra, staining mechanism and detection capabilities available (confocal laser scanning microscope and microplate reader equipped with the following filter cubes: DAPI, GFP, RFP and Texas Red). Of these fluorophores, the best combination was Hoechst 33342 and SYTOX® Orange with the Hoechst 33342 staining all cells and the SYTOX® Orange only the dead cells, leaving the red channel for endosymbiont detection and the green channel for endogenous GFP (Fig. 4, see **Material & Methods** for details). The outcomes of staining for all fluorophores tested are summarized in Table 1.

As described in Table 1, several established live/dead stains are ill-suited to coral cells because of spectral overlap with endogenous signals. When using bandpass filters, the emission from the cell impermeant stains propidium iodide and ethidium homodimer cannot be separated from endosymbiont chlorophyll fluorescence to mark dead cells. The nucleic acid stain SYTOX® Orange (λ_{ex} 547 nm, λ_{em} 570 nm), also a cell impermeant stain, penetrates cells with compromised plasma membrane and has an emission wavelength further away from that of chlorophyll. The SYTOX® line of dead stains is available in different colors, which could be useful in coral samples with emission spectra near 570 nm, as reported for number of Acroporidae, Poritidae, and Faviidae corals (Fig. 1).

Cytotoxicity assay

In order to test the cytotoxicity of titanium dioxide nanoparticles (TiO_2 NPs) and insulin, cell mixtures collected from *P. damicornis* were plated on 96 well-plates coated with poly-D-lysine at a density of ~ 400,000 cells per well with complete medium. After a 1 hour rest period, the cells were exposed to

concentrations of insulin and TiO_2 NPs ranging from 0.5 to 100 µg/mL in culture medium. Triton X-100 was used as positive control. Cell survival was measured after 4 hours of exposure using the MTS and LDH assays and after 24 hours of exposure using the Hoechst 33342 – SYTOX® Orange dye pair.

Two colorimetric assays were tested here: the MTS and LDH assays. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and LDH (lactate dehydrogenase) assays are commonly performed in combination. Although reliable and simple, limitations of the MTS and LDH assays are that serum and other compounds in the culture media can introduce distortions or background[15,16].

The MTS assay did not yield satisfactory results after 1 hour at 25°C, the incubation time recommended for mammalian cells. The expected reaction is a color change from the yellow MTS tetrazolium as it is converted by cell metabolism to the purple/brown formazan. No color change was also measured with longer incubation times of 6, 12, 24 and 48 hours at 25°C. The absorbance measurements of the failed MTS assays are not presented. The LDH assay successfully measured the presence of lactate dehydrogenase in the media taken from the exposed *P. damicornis* cell cultures. However, the results were variable and the media-only control was not consistent throughout the experiments (**Supplementary Materials S.2**).

The measurements based on the Hoechst 33342 – SYTOX™ Orange dye pair showed TiO_2 NPs reduces *P. damicornis* cell viability at concentrations above 10 µg/mL with a calculated lethal concentration 50% (LC50) of 20 µg/mL after 24 hours of exposure. Insulin also reduced *P. damicornis* cell viability around the same extent for concentrations between 10 and 100 µg/mL after 24 hours of exposure (Fig. 5). Statistical analysis (two-way ANOVA with blocking and Tukey post-hoc, see **Materials and Methods**) revealed significant differences between the control (0 µg/mL) and concentrations above 1 µg/mL for all treatment (**Supplementary Materials S.2**). Significant differences were also found between the positive control treatment (Triton X-100) and TiO_2, and insulin, confirming its efficacy as positive control. No significant difference was found between the TiO_2 and insulin treatments (Fig. 5, **Supplementary Materials S.2**)

Discussion

Mammalian cell-based assays are poorly adapted to *in vitro* work with scleractinian coral cells. This is in part due to the diversity and abundance of endogenous fluorescence present in reef-building corals but also due to factors such as the lack of cell attachment, the salinity (~ 35‰) or strong ionic (~ 0.7 M) nature of seawater, and other unknowns. Indeed, as our knowledge of coral cell physiology and function grows, so will the diversity of coral-specific assays. Coral cell viability assessment is key to developing other assays as cell viability is one of the most straightforward endpoints of *in vitro* research. In this study we developed a framework for coral scientists to tailor fluorescence-based membrane integrity assays to the coral species phenotype of their choice. This method first involves accurately determining the different fluorescent signals emitted by the coral species genotype and finding fluorescent dyes that

do not overlap or that can easily be deconvoluted. The membrane integrity-based dye pair Hoechst 33342-SYTOX® Orange, avoids the endogenous fluorescent signals of *P. damicornis* cells and allows us to test the toxicity of TiO_2 and insulin *in vitro* applied to cells dissociated for scleractinian coral *P. damicornis* (green phenotype) for the first time. Two viability assays common in mammalian research, the MTS and LDH assays, were also tested yielding unsatisfactory results. The poor reaction observed with the MTS assay can potentially be explained by the fact that, for the reduction of tetrazolium salt to occur as a function of cellular activity, reductase need to be transported across the plasma membrane into the culture medium which is not common to all organisms[17]. The inconsistencies in the LDH assay results on the other hand, might be dur to the presence of other dehydrogenases, such as opine dehydrogenases which are functionally analogous to lactate dehydrogenase, being used to regenerate NAD^+ in many invertebrates[18]. *Montipora capitata*, another scleractinian coral, showed strombine dehydrogenase and alanopine dehydrogenase activity but no LDH activity[18].

One of the complications associated with *in vitro* coral research is limited cell attachment. Compared to adherent mammalian cell lines which attach to the substrate, coral cells have shown limited attachment to standard culture flasks and plates[3]. Here, we worked with mixed, unsorted cells; therefore, we increased cell attachment by coating the well plates with poly-D-lysine to reduce cell loss during aspiration. Without poly-D-lysine, cell counts dropped drastically and reached levels below critical mass. Poly-D-lysine had the side effect of trapping the TiO_2 nanoparticles which resulted in the persistence of TiO_2 agglomerates after exposure medium washing, which is visibly present in the brightfield images. The entrapment of TiO_2 particles could have reduced their interactions with *P. damicornis* cells and affected the measured cytotoxicity. TiO_2 nanoparticles have strong UV photoactive properties which have led to their increased use in paints, solar cells, and sunscreens and consequently their unintentional release in the environmentt[19]. The toxicity of TiO_2 nanoparticle exposure in various freshwater (reviewed in [20]) and marine organisms (reviewed in [21]) has been tested, and the toxicity has been shown to increase with UV exposure due to photoactivation[20,21]. It is important to note that UV conditions are not always reported making it challenging to determine UV-enhanced cytotoxicity. The light requirements of reef-building corals intrinsically involve UV exposure, and we applied a 10 hour light / 14 hour dark cycle (PAR 40 ± 2, i.e. ~ 8.71 W/m^2 = 0.0871 mJ/cm^2, see **Materials and Methods** for more details) throughout our experimentation. TiO_2 has been shown to damage symbiotic dinoflagellates and induce bleaching in *Acropora* spp. corals[22] and *Montastraea faveolata*[23], and also to increase mortality, abnormal movements and abnormal feeding behaviors in clown fish[24], and reduce marine phytoplankton growth[25]. In both *Acropora* and *Montastraea*, slight bleaching occurred after exposure to 6.3 mg/L TiO_2 for up to 48 hours and 10 mg/L TiO_2 for 17 days. Our results corroborate these findings with a reduction in cell viability starting at 10 mg/L TiO_2 with LC50 at 20 mg/L under a UV intensity five times lower than that experiences in the environment. Environmental concentration of TiO_2 are estimated to range between 0.02–0.9 mg/L of TiO_2 per day based on a study performed along three beaches in the south of France[26].

These concentrations are orders of magnitude less than the LC50 found here; however, studies to date including ours represent only short term exposures.

The symbiotic mutualism between coral host and dinoflagellate endosymbionts fulfills up to 90% of the holobionts energetic needs. This energy trafficking suggests a transport and signaling system where a molecule such as insulin could come into play. Furthermore, as bleaching involves the breakdown of symbiosis, this transport and signaling system can be disrupted, presumably with similarities to the diabetic response in vertebrates. To enable future investigation of this hypothesis, we here investigated insulin cytotoxicity as an example for proteotoxicity. Insulin has been a model system for the study of proteotoxicity in protein evolution[27], and different conformations and oligomerization states of insulin are relevant in the etiology and treatment of diabetes[28,29]. There are numerous reports of preproinsulin-like psudogenes in a variety of different organisms including insects, invertebrates, plants and microbial eukaryotes and prokaryotes[30]. Remarkably, human insulin has been shown to have physiological effects on other organisms, such as *Acanthamoeba castellanii*[31], suggesting a conservation of structure and function across long evolutionary distances. Thus, the natural first step in evaluation the effects of insulin on *P. damicornis* is its potential cytotoxicity. Our findings suggest that insulin reduces *P. damicornis* cell viability (~ 20% decrease) at concentrations between 10 and 100 µg/mL. Insulin cytotoxicity is known to depend on different solvent properties, such as increased temperature and high concentrations of salts[32,33], leading to insulin aggregates and misfolding. The salinity or the ionic strength of seawater could have had similar effects despite the relatively low temperature (25°C) and basic nature of the culture medium. Thus, it is possible that seawater might be modifying insulin conformation and cytotoxic behavior, and the present work lays the foundation for further research related to the effects of insulin on corals.

Regardless of organism studied, the type of cell death mechanism is highly dependent on the nature and duration of the stress applied and the ability of cells to maintain homeostasis. The positive control, Triton X-100, is a common surfactant used to lyse cells. It is cytotoxic to a number of cell types: ciliated protozoan, fish and mammalian[34]. Using it as a positive control for membrane integrity assays involves the understanding that cell count will decrease with increasing Triton X-100 concentration. Different mechanisms of cell death can be involved when coral cells are exposed to substances such as insulin and TiO_2 NPs tested here. Therefore, the Triton X-100 serves as a positive control verifying that the assay worked and additional research is needed to identify the mechanisms of cell death. For example, in mammalian cells, the absence of caspase activation, cytochrome c release, DNA fragmentation, membrane damage and changes in cell morphology are all cell parameters that can be used to discriminate cell necrosis from apoptosis[35] and other types of cell death. This distinction is also particularly relevant in the study of dysbiosis. The breakdown of symbiosis between endosymbiotic dinoflagellate algae and coral host is still not well understood despite the urgent need to characterize coral bleaching at the cellular level. Stable cultures of the endosymbiont-holding gastrodermal cells, combined with cytotoxicity and cell death mechanism assays could help better define the mechanisms of coral-dinoflagellate dysbiosis.

Conclusion

The global loss of coral cover driven by anthropogenic climate change has underscored the need for a better understanding of coral cell biology. Research progress in this area will be driven by the optimization of marine invertebrate cell culture methods and the potential transfer of *in vitro* methods developed for model organisms. Cell viability is a key measurement, and development of membrane integrity assays for coral cell culture represents an essential step in the advancement of *in vitro* coral research. Our results show that the Hoechst-SYTOX dye pair is well suited for membrane integrity assessments in *P. damicornis* cells. This dye pair was used to measure quantitatively the toxicity of Triton X-100, TiO_2 and insulin in coral cells for the first time *in vitro*. These results open the door to in-depth, quantitative evaluation of toxicity of different reagents on corals at the cellular level.

In addition, further development of cellular assays will provide new tools to better understand cnidarian cellular physiology, transmembrane exchanges and cell death mechanisms.

Methods

Coral cell dissociation and culture

Fragments of *Pocillopora damicornis* (ORA Aquaculture purchased from Live Aquaria, FL) were maintained in 37.8L aquaria and supplied with oxygenated artificial seawater (ASW, constant bubbling, reverse osmosis deionized water [5 Stage Premium RO/DI water saver system from Bulk Reef Supply] + Fritz Reef Pro Mix salts, SG 1.025 ± 0.002, pH 7.5-8, 25°C ± 1°C, pump flow rate: 378.5 L/H, pump filter: activated carbon Aqueon, 10h-light/14h-dark cycle AI Prime® 16HD Reef light: blue 28%, royal 28%, green 28%, deep red 28%, UV 28%, violet 28%, cool white 28%, moonlight 28%, PAR 40 ± 2). General weekly maintenance included tank cleaning to remove macroalgae overgrowth, coral feeding (live Artemia: Carolina® brine shrimp eggs) and replacing 2L of seawater.

The cell dissociation protocol follows that reported in Roger et al.[3] Briefly, a coral nubbin (*P. damicornis*) of ~ 5 mm length was cut using sterile clippers and placed in artificial seawater (RO/DI H_2O + Fritz Reef Pro Mix) with Reef Dip™ coral disinfectant (25 µL/mL of seawater) under constant bubbling for 10 min. The nubbin was then rinsed with sterile filtered, autoclaved ASW 3 times and incubated in calcium- and magnesium-free ASW (autoclaved and sterile filtered) for 1 hour in a biosafety cabinet under ambient light. The nubbin was then washed using the solution in the vial to detach remaining cells and maximize cell count. The cell suspension was centrifuged at 1200 rpm (204 RCF) for 3 min at 25°C and the supernatant replaced with complete culture medium (CM). The complete CM is composed of 15% Dulbecco's Modified Eagle Medium (without phenol red, with 17.491M D-glucose, 2.50 mM L-glutamine) + 5% Fetal Bovine Serum + 0.5% antibiotic-antimycotic + 0.5% gentamicin + 79% filtered sterile ASW).

TiO_2 and insulin exposures

Dissociated coral cells in complete coral CM were plated in poly-D-lysine coated 96 well plates at a density of ~ 400,000 cells per well. Poly-D-lysine (ThermoFisher cat. No. A3890401) coated 96 well plates were prepared following the manufacturer's protocol. Initial viability was assessed using a 1 mL aliquot of the cell suspension stained with the Hoechst 33342/SYTOX® Orange live/dead staining solution and counted on a disposable hemocytometer (INCYTO C-Chip, Neubauer Improved format) prior to platting. Following a 1 hour rest period, cells were incubated in coral CM alone (negative control) or coral CM containing serial dilutions (0.5, 1, 5, 10, 50 and 100 µg/mL) of Triton X-100 (positive control, Sigma-Aldrich cat. no. T8787), insulin (human recombinant (yeast), MilliporeSigma cat. no. 11376497001), or TiO_2 nanoparticles (nanopowder, 21 nm primary particle size, Sigma-Aldrich cat. no. 718467, characterization data presented in **Supplementary Materials S.3**) for 24 hours at 25°C. Triplicate wells were exposed for each dose and the assay was performed three times. After exposure, the plates were centrifuged at 1200 rpm (204 RCF) for 3 minutes at 25°C. The medium was replaced by the Hoechst 33342/SYTOX® Orange live/dead staining solution and incubated in the dark for 30–60 minutes. After staining the plates were centrifuged at 1200 rpm (204 RCF) for 3 min at 25°C. The staining solution was aspirated and fresh coral CM was added to each well before imaging. Photos of each well (4x magnification) were taken using a Cytation3 microplate reader mounted with 4 filter cubes (BioTek Agilent): DAPI (λ_{ex} 350–405 nm, λ_{em} 415–480 nm), GFP (λ_{ex} 445-490nm, λ_{em} 501–550 nm), RFP (λ_{ex} 505-560nm, λ_{em} 520–570 nm) and Texas Red (λ_{ex} 575–600 nm, λ_{em} 510–585 nm). Cells were counted in each photo and percent viability post-exposure was determined by [(Hoechst positive cells - SYTOX® positive cells)*100/(Hoechst positive cells)]. Cell counting was performed using BioTekGen5 software automation in 1000 x 1000 µm squares in each photo (five photos were taken in each well).

Live/Dead assay

Two fluorescent dyes were selected: Hoechst 33342 (20 mM, ThermoFisher cat. no. 62249) and SYTOX™ Orange (250 µM solution in DMSO, ThermoFisher cat. no. S34861). To a 1 mL coral cell suspension, 10 µL of SYTOX™ Orange (0.2 µM final concentration) and 2 µL of Hoechst 33342 (40 µM final concentration) were added. Hoechst 33342 and SYTOX™ Orange are both DNA-binding dyes; however, Hoechst 33342 is membrane permeable whereas SYTOX™ Orange is not. In other words, Hoechst 33342 stains all cells while SYTOX™ Orange stains only cells with damaged membranes. Cells were exposed to the staining solution for 30–60 minutes and washed with fresh complete coral CM before imaging.

MTS and LDH assays

Dissociated coral cells were plated in 96 well plates at a density of ~ 250,000 cells per well in complete coral CM. After a 1 hour rest period, initial viability was assessed using a small aliquot of the cell suspension stained with the Hoechst 33342/SYTOX® Orange live/dead staining solution and counted on a disposable hemocytometer prior to plating. Cells were incubated in coral CM alone (negative control) or coral CM containing serial dilutions (0.5, 1, 5, 10, 50 and 100 µg/mL) of Triton X-100 (positive control), insulin, or TiO2 NPs for 4 hours at 25°C. Triplicate wells were exposed for each dose and the assay was performed three times. After exposure, the plates were centrifuged at 1200 rpm (204 RCF) for 3 min at

25°C. A 50 µL volume of supernatant from each well was transferred to a white 96-well plate and left to equilibrate to room temperature for 20 minutes. Then, 50 µL of CytoTox-ONE™ reagent (CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit, Promega, G7891) was added to each well and incubated at room temperature for 10 minutes. After adding 25 µL of Stop Solution to each well, the fluorescence intensity was measured at λ_{ex}560/λ_{em}590 nm using a microplate reader (Cytation 3, BioTek).

The remaining exposure medium was removed from the wells and 120 µL of MTS reagent was added to each well. The MTS reagent consisted of 7.1 mL of coral CM and 1.4 mL of MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay kit, Promega, G3580). The plate was incubated at 25°C for 1 hour in the dark. Following incubation, the absorbance was measured at 490 nm using a microplate reader.

Statistical analysis

The cell viability data was analyzed using a two-way ANOVA with blocking to consider variability between replicate assays (viability ~ concentration + assay + molecule), and Tukey post-hoc (95% family-wise confidence level). All analyses performed and results can be found in **Supplementary Materials S.2.**

Microscopy

Single optical sections of coral cell suspension were acquired with a Zeiss LSM 880 or LSM 710 confocal microscope, build on Axio Observer Z1 inverted stand, equipped with motorized stage and a 40x Plan Apo oil immersion objective (NA 1.4). Fluorescence was excited with either one or a combination of lasers available on the system: 405 nm diode (15 mW), 440 nm diode (15 mW), multiline Ar ion (458/488/514 nm, 25 mW), 561 nm DPSS (15 mW), 594 nm and 633 nm He-Ne (3 mW each). Excitation power was adjusted between 0.8% and 2%, depending on the line. Fluorescence spectrum was registered with a 32-channel spectral hybrid detector at the gain of 500V. Each channel corresponded to 7.9 nm. The pinhole was set to 1.25 Airy units at 550 nm emission. Transmitted light (DIC) was registered together with fluorescence. Images were collected in tile-based mode (5x5), with total area of 1220 x 1220 um, 0.13 um pixel size and 2.05 us dwell time. All signals were digitized with 16-bit precision.

Declarations

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Additional information

The authors declare that no competing interests exist.

Supplemental Materials

S.1. Spectral scans performed on *Pocillopora damicornis* live cells with seven different lasers: 405 nm (**A**); 440 nm (**B**); 458 nm (**C**); 488 nm (**D**); 514 nm (**E**); 561 nm (**F**); 633 nm (**G**). (Zeiss LSM 710, VCU Microscopy Core): DOI 10.17605/OSF.IO/69JPX

S.2. Cytotoxicity data and statistical analysis

S.3. Transmitted Electron Microscopy image of Titanium dioxide (TiO_2) powder.

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Supplemental Materials

Supplemental Material S.1. is not available with this version

Figures

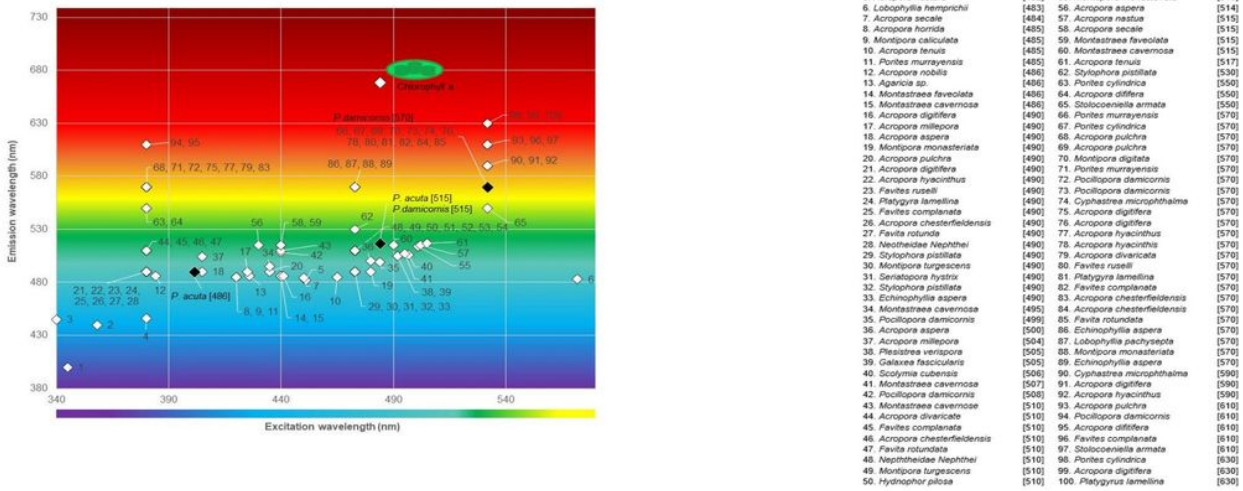

Figure 1

100 Coral chromophores. Selection of 100 coral chromophores excitation ($\boldsymbol{\lambda}_{ex}$) and emission ($\boldsymbol{\lambda}_{em}$) wavelengths reported in 10 different coral families (43 scleractinian coral species) and measured in *Pocillopora damicornis* (this study). Coral species are listed with number key corresponding to the graph and [emission wavelength] detected (and reported in literature). References are as follow: (1-3, 8-9, 11, 17, 20, 35-36, 38)[36]; (4)[37]; (5, 7, 10, 19, 55-57, 61)[38]; ; (6)[39]; (12)[40]; (13)[14]; (14-15, 58-59)[41]; (16, 60)[42]; (18, 37)[43]; (21-33, 44-54, 62-100)[44]; (39)[45]; (34, 41)[12]; (40)[13]; (42)[46]; (43)[47].

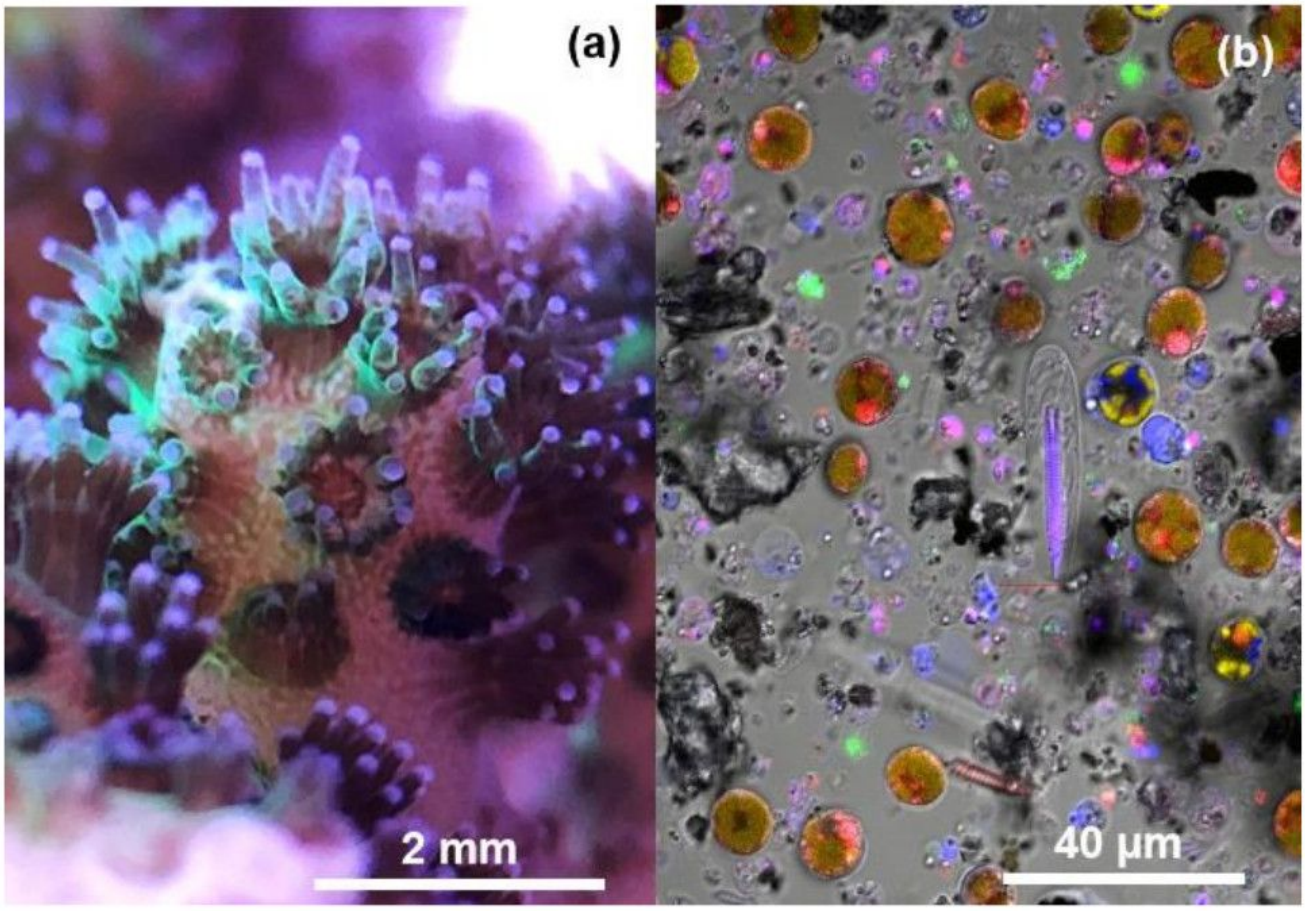

Figure 2

Photograph of *Pocillopora damicornis* in aquarium with polyps displaying the green fluorescent phenotype (a) and cells dissociated from *P. damicornis* stained with ReadyProbes™ cell viability imaging kit (red: propidium iodide for dead cells, blue: NucBlue® for all cells) imaged under confocal laser scanning microscope (Zeiss LSM 710, VCU Microscopy Core)[other colors are as follows: yellow for chlorophyll fluorescence and green for endogenous GFP, DIC set for contrast on center cell] (b). The most recognizable cell types are the symbiotic dinoflagellates (golden-brown rounded cells, color generated from overlay of yellow and red) and the nematocysts (oblong cells with bottle brush-like internal structure). Other cell types cannot be identified from morphological characteristics.

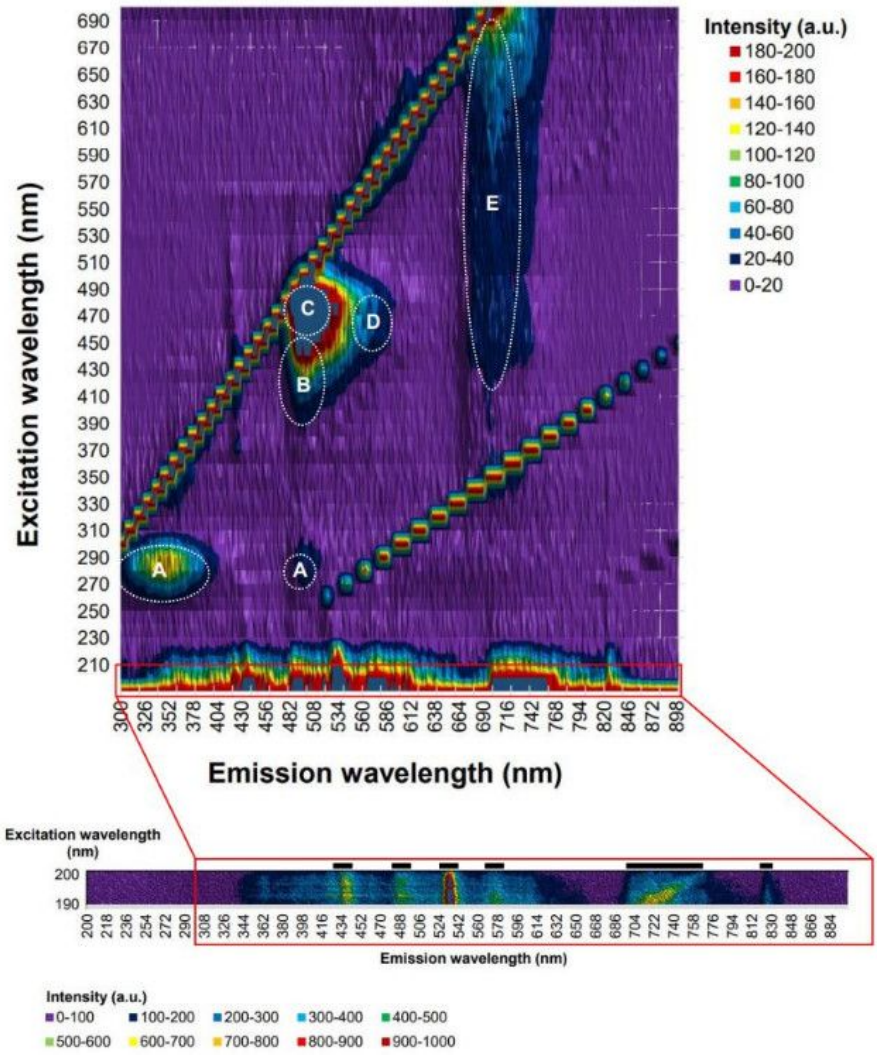

Figure 3

Excitation-emission matrix for *Pocillopora damicornis.* Five emission peaks are present: tryptophan (A), cyan fluorescent protein (B), green fluorescent protein (C), red fluorescent protein (D) and chlorophyll (E). Insert shows high resolution fluorescence matrix under deep UV excitation (190 to 200 nm), which also contains the same five emission peaks. Note the chlorophyll fluorescence presents a wide emission band from ~700nm, which is well documented[48].

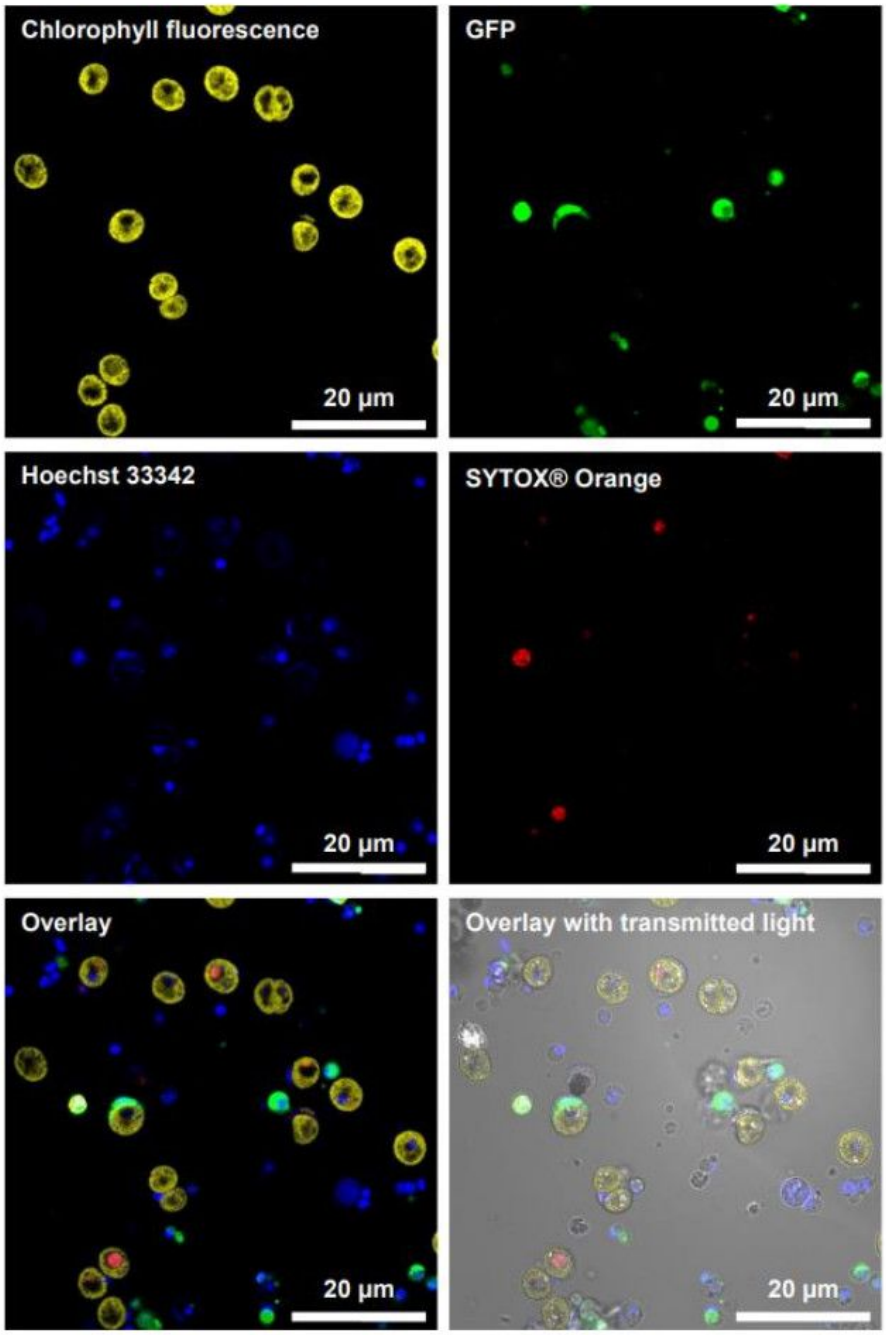

Figure 4

Live cell confocal fluorescence laser scanning microscopy images of *Pocillopora damicornis* cells (Zeiss LSM 710, VCU Nanomaterial Characterization Core). Cells were co-stained with SYTOX® Orange (0.2 µM) and Hoechst 33342 (40 µM) for 30 minutes. Magnification = 63x.

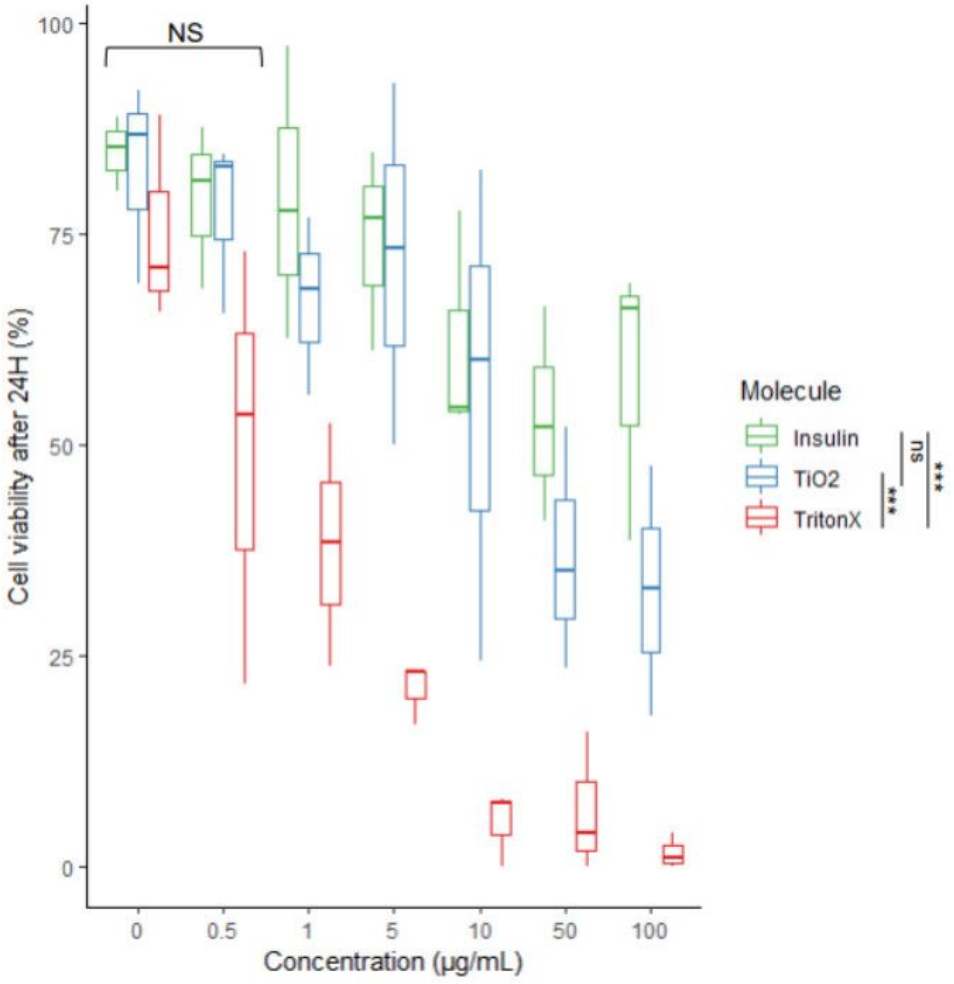

Figure 5

Coral cell viability after 24 hours exposure to concentrations of Triton X-100 (positive control, cell lysate), titanium dioxide (TiO_2) and insulin between 0.5-100 µg/mL. The boxes summarize the data with median (bold line) and outliers (whiskers). The different molecules tested yielded statistically significant results

between 1-100 µg/mL but not between 0-0.5 µg/mL (NS). The control used (Triton X-100) yielded significantly different results compared to TiO_2 and insulin, confirming its role as positive control. Further statistical analyses can be found in **Supplementary Materials S.2.**

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- S2.Cytotoxicitydataandstats.csv
- SupplementalMaterialslegends.docx
- S.3.TEMoftitaniumdioxidenanoparticles.pdf
- S.1.Linktospectralfiles.docx