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Optical hyperthermia mediated by Gold Nanoprisms to Boost Hydra Regeneration: Insights into Calcium Dynamics

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Abstract: This study explores the usage of gold nanoprisms (AuNPs) as intracellular nanoheaters to enhance *Hydra* regeneration via NIR irradiation. It investigates local and global effects of heat on cellular processes, employing advanced imaging techniques to track calcium fluxes.

1. INTRODUCTION

Optical hyperthermia mediated by plasmonic nanoparticles is a non-invasive technique enabling a controlled temperature increasing in biological tissues. Numerous studies suggest the use of heat not only for inducing specific cell ablation but also in regenerative medicine, where small doses of heat can modulate wound healing and tissue regeneration. Photothermal agents, such as gold nanoprisms (AuNPs), have been used as "nano-hotspots" to selectively generate heat in a spatiotemporal manner [1]. To assess the potential of AuNPs as optothermal actuators for tissue regeneration, it is essential to have a comprehensive understanding of their *in vivo* behavior and their ability to modulate cellular functions in response to thermal stimulation. To this end, we propose *Hydra vulgaris* as a model organism due to its extraordinary ability to regenerate amputated body parts. Photostimulated AuNPs in *Hydra* have been shown to induce various responses, simply by modulating their photothermal properties [2,3]. In this study, we investigate the possibility of enhancing *Hydra* regenerative potential by reactivating stem cell self-renewal through heat delivery mediated by gold nanoparticles. By conducting analyses at morphological, molecular, and cellular levels, we observed an increase in *Hydra* regenerative potential, attributed to the reactivation of stem cell self-renewal following treatment with NIR light-photostimulated nanoparticles. To better understand how the heat generated by AuNPs enhances *Hydra* regeneration, we employ advanced imaging techniques, Light-Sheet Fluorescence Microscopy (LSFM), using a transgenic *Hydra* expressing a calcium reporter. LSFM will be equipped with an irradiation system enabling simultaneous perform AuNP/NIR irradiation and the detection calcium signals, mapping calcium fluxes during regeneration.

2. RESULTS

2.1 Cellular and molecular events evoked by AuNP photostimulation in regenerating polips

To understand the impact of AuNPs on *Hydra* regeneration, we amputated *Hydra*'s head and monitored the regeneration for 72 hours. We compared the effect of a macroscopical external source (28°C), versus nanometric

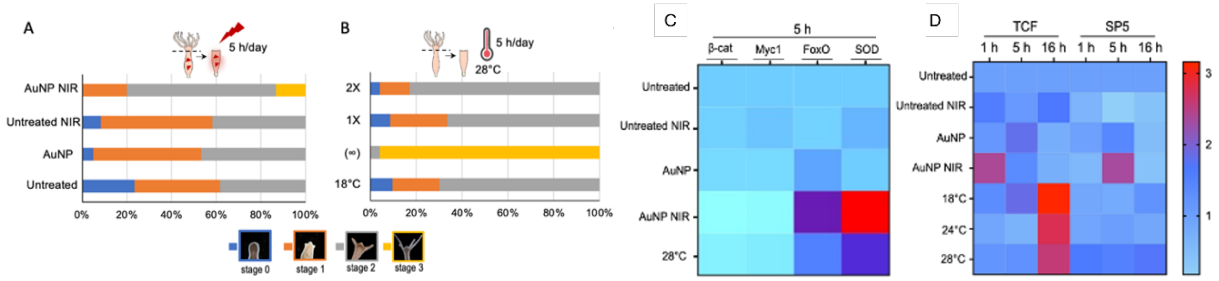

Fig 1. Regeneration assay and heat map of gene expression analyses. A) Polyps treated with AuNPs (1mg/ml), bisected and irradiated with NIR light. The percentage of regenerating heads has been scored every 24 h based on their developmental stage. B) Polyps bisected and exposed for different conditions to 28°C for 5 h /day. 1X = one pulse of heat (28°C), 24 h p.a.; 2X = two pulses of heat, 48 h p.a. Three independent biological replicates have been performed (n = 60). C) Gene expression levels by qRT-PCR modulated during regeneration, 5 h post head amputation. D) Time-course of *TCF* and *Sp5* during regeneration after AuNP-NIR treatment and at 28°C. Data are the average of three biological replicates, n=15, and three technical repeats.

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intracellular source (AuNPs). We analyzed four conditions: Untreated, Untreated NIR, AuNPs and AuNP-NIR. The regenerative process in *Hydra* is well known: 30-36 hours after amputation, small tentacle appears at the regenerating tip (stage 1). In the following 24 hours the tentacles elongate (stage 2), completing their growth in 72 h (stage 3). The Fig.1A shows the significant increase of regeneration stages 2 and 3 in the animal treated with AuNPs and NIR irradiated, as observed at 28 °C (Fig. 1B).

To assess if the increased regeneration and reproduction in photostimulated polyps is correlated positively with the rate of cell proliferation, we performed a $BrdU^+$ assay. $BrdU^+$ assay revealed a significant increase in stem cell proliferation after treatment with AuNPs combined with NIR irradiation or exposure to 28 °C for 5 hours. To understand the molecular controllers involved in *Hydra vulgaris* self-renewal and differentiation, we investigated the key transcription factors *forkhead box (FoxO)* [4], β-catenin (*β-cat*), the proto-oncogene *Myc1* and Superoxide dismutase (*SOD*) [5,1]. Five hours after AuNP-NIR irradiation, *β-catenin* and *Myc1* were significantly downregulated, suggesting their role in regulating interstitial stem cell (ISC) self-renewal. Meanwhile, *FoxO* and *SOD* were upregulated, consistent with the known role of *FoxO* in self-renewal (Fig.1C). According to these data, we hypothesized that the increase in regeneration efficiency is correlated with a temporal advance in the expression of genes involved in *Hydra* regeneration and development. Thus, we analyzed two genes: T-cell factor (*TCF*) and *Sp5* which activated in the initial phase of *Hydra*'s head regeneration [6,7]. The results showed that the improved regeneration may depend on a temporal acceleration of *TCF* and *SP5* expression in AuNPs irradiated animals, as occurs at 28°C (Fig.1D).

2.2 Setup up for Functional Imaging of Calcium Dynamics Using LSFM

Functional imaging to visualize calcium variations in *Hydra* was performed using a LSFM system similar to that described in [8]. In the current setup, LSFM employs Olympus UMPLFLN 10XW objectives (NA = 0.3) for both illumination and detection. The specimen was mounted in FEP tubes ranging from 0.4 to 0.8 mm in diameter, fully immersed in its native medium without the use of anesthetic agents. The animal was subjected to amputation, with the head region removed, and was subsequently observed at 18 and 42 hours post-injury. Image stacks consisting of 20-30 frames spaced 10 µm apart were acquired every 3-4 seconds for 10 minutes. Figure 2A presents maximum intensity projections (MIP) of a sequence of 13 consecutively acquired volumes at 18 hours post-amputation, demonstrating the limited calcium oscillations or spikes (Fig. 2C). Conversely, at 42 hours post-amputation (Fig. 2B), a series of consecutive acquisitions revealed the reestablishment of contraction-associated calcium oscillations, as quantified in the fluorescence analysis (Fig. 2D). This technique enables the investigation of how tissue regeneration impacts ectodermal circuit activity. Moreover, single-cell resolution imaging highlights that ectodermal cell activations are neither uniform nor simultaneous across the sample, suggesting cell-to-cell communication and regional specificity. Figure 3E illustrates a portion of the tissue during contraction, where a calcium wave propagates from one cell to another, as noticeable from the graph in Fig 3F. Future applications of this system will include an in-depth screening of the effects of regeneration on the *Hydra* ectoderm, particularly in response to thermal stimuli. These stimuli will be delivered using a 1064 nm laser integrated with the LSFM system, in conjunction with AuNPs.

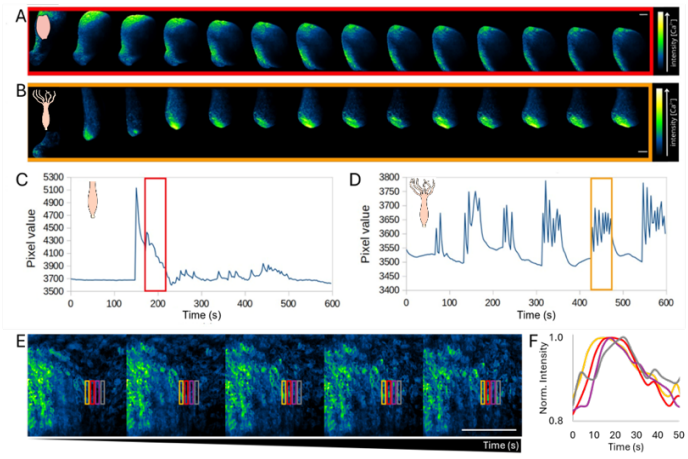

Fig. 2. LSFM acquisitions of freely behaving *Hydra*. A) MIP of volumetric timelapse images (Δt=3s) of *Hydra* performed 18 h post amputation. B) MIP of volumetric timelapse images (Δt=3s) of *Hydra* performed 42 h post amputation. C) Calcium signal along time over the whole field of view of (A). D) Calcium signal along time over the whole field of view of (B). E) MIP of volumetric timelapse images (Δt=6s) of *Hydra* during contraction, showing a calcium wave-like activation of tissue. F) Calcium signal extracted from the colored boxes in (E). Scale bars are 200 µm.

3. CONCLUSIONS

Here, we demonstrate the possibility of using plasmonic nanoparticles to control a fundamental biological process, tissue regeneration, by the simple application of light. Studying the cellular and molecular mechanisms by which animals regenerate missing body parts is of primary interest for improving our understanding of tissue/organ regeneration in humans and may help identify novel strategies to restore lost regenerative capacity. Our results shed light on a novel function of heat-emitting nanoparticles to control cell stemness through the activation of molecular pathways that could be targeted for regenerative medicine or wound-healing strategies. Furthermore, analysis of calcium oscillations revealed that post-amputation regeneration is associated with ectodermal cell activity, suggesting cell communication and regional specificity.

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