Cumhuriyet Sci. J., 45(2) (2024) 360-365
DOI: https://doi.org/10.17776/csj.1462654

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Multispectral Imaging and Single-Cell Analysis with Genetically Encoded Biosensors Unveil Complex Interactions between Extracellular ATP and Intracellular Calcium

Emrah Eroglu [1,2,a,*]

[1] *Molecular Biology, Genetics and Bioengineering Program, Faculty of Engineering and Natural Sciences, Sabanci University, Istanbul, Türkiye.*
[2] *Research Institute for Health Sciences and Technologies (SABITA), Istanbul Medipol University, Istanbul, Türkiye.*
**Corresponding author*

Research Article

History
Received: 01/04/2024
Accepted: 07/06/2024

ABSTRACT

The dynamic interplay between extracellular ATP (eATP) and intracellular calcium ($[Ca^{2+}]_i$) serves as a pivotal signaling axis in cellular physiology, influencing a myriad of cellular processes. Traditionally recognized as an energy currency within the cell, ATP has emerged as a multifunctional signaling molecule that orchestrates diverse cellular responses through activation of purinergic receptors. The complex link between ATP signaling and calcium dynamics plays a central role in cellular communication and homeostasis. Advancements in imaging technologies such as development of genetically encoded biosensors have revolutionized the study of cellular signaling dynamics, enabling visualization of the spatiotemporal aspects of eATP and $[Ca^{2+}]_i$ in real-time. The convergence of eATP signaling and $[Ca^{2+}]_i$ dynamics serves as a central hub in cellular communication. In this study, utilizing bicistronic construct biosensors for multispectral imaging of $[Ca^{2+}]_i$ responses to eATP, we show that distinct concentrations of eATP administration reveal complex intracellular $[Ca^{2+}]_i$ responses, potentially attributed to receptor desensitization. Single-cell co-imaging uncovers $[Ca^{2+}]_i$ heterogeneity, emphasizing the significance of individual cell dynamics in eATP-induced calcium signaling. Therefore, this study sheds light on the intricacies of eATP-induced calcium signaling, providing insights valuable for basic research and therapeutic applications.

Keywords: Calcium signaling, ATP signaling, Single cell imaging, Genetically encoded eiosensors, Multispectral imaging.

[a] *Emrah.eroglu@medipol.edu.tr* *https://orcid.org/0000-0002-9373-0808*

Introduction

The dynamic interplay between extracellular ATP (eATP) and intracellular calcium ($[Ca^{2+}]_i$) serves as a pivotal signaling axis in cellular physiology, influencing a myriad of cellular processes ranging from cell proliferation to synaptic transmission [1,2]. ATP, traditionally recognized as an energy currency [3,4] within the cell, has emerged as a multifunctional signaling molecule that orchestrates diverse cellular responses through activation of purinergic receptors [5,6]. Notably, the intricate link between ATP signaling and calcium dynamics plays a central role in cellular communication and homeostasis [7,8].

Adenosine triphosphate (ATP), primarily known for its role in cellular energy transfer, has garnered increasing attention as an extracellular signaling molecule [9]. The recognition of purinergic receptors, encompassing P1 adenosine and P2 purinoceptor families, has established ATP as a key mediator in intercellular communication [10]. Among the P2 purinoceptor family, P2X receptors directly respond to eATP, leading to the influx of calcium ions and subsequent activation of downstream signaling cascades [11]. The coupling of eATP to intracellular calcium signaling is integral for the regulation of cellular responses [12,13], making it a focal point for investigations aimed at unraveling the complexities of cellular communication.

Advancements in imaging technologies have revolutionized the study of cellular signaling dynamics [14], enabling researchers to visualize the spatiotemporal aspects of eATP and $[Ca^{2+}]_i$ in real-time [15-17]. Chemical and genetically encoded biosensors have played pivotal roles in this endeavor [18]. Traditional chemical indicators, such as Fluo-4 and Fura-2, offer high sensitivity but are limited by issues like photobleaching and potential cytotoxicity [19]. Genetically encoded biosensors, on the other hand, provide a versatile and targeted approach by fusing fluorescent proteins with proteins sensitive to specific signaling molecules [18].

This study, employed a bicistronic construct expressing $GRAB_{ATP1.0}$ and RCaMP, genetically encoded biosensors for eATP and $[Ca^{2+}]_i$, respectively [20]. This approach not only allows for the simultaneous monitoring of extracellular ATP and intracellular calcium but also offers the advantage of improved spatial resolution and reduced perturbation compared to traditional chemical indicators. The specific binding and fluorescence changes associated with these genetically encoded biosensors offer a direct and reliable readout of eATP and $[Ca^{2+}]_i$, facilitating a more comprehensive understanding of the signaling events.

While genetically encoded biosensors have significantly advanced live-cell imaging, the importance of multiparametric imaging cannot be overstated [21]. Multispectral imaging involves the simultaneous

acquisition of multiple wavelength bands, allowing for the discrimination of distinct fluorophores [7,22,23]. This study utilizes the $GRAB_{ATP1.0}$ [15] and RCaMP [22] permitting multispectral imaging to capture their unique spectral properties using conventional widefield microscopes, a common challenge in conventional imaging techniques. This approach provides a nuanced perspective, enabling the separation of signals from different probes, thereby refining the accuracy of data interpretation. In the context of eATP and $[Ca^{2+}]_i$ imaging, multispectral analysis would not only improve the reliability of signal detection but also facilitate the identification of potential crosstalk or interference between the two signaling pathways. Thus, the incorporation of multispectral imaging with genetically encoded biosensors is instrumental in advancing the precision and discriminatory power of live-cell imaging studies.

The cellular landscape is inherently heterogeneous, with individual cells within a population often exhibiting diverse characteristics [24]. Single cell heterogeneity is particularly evident in signaling pathways, where cellular responses to external stimuli can vary significantly even within clonal cell lines [25]. Traditional population-level measurements may obscure critical insights into the underlying dynamics of cellular signaling [26]. Single cell imaging techniques provide a powerful tool to unveil this heterogeneity and offer a more granular understanding of cellular responses [27]. In the context of our study, single cell imaging allowed us to discern variable $[Ca^{2+}]_i$ responses despite consistent eATP signals. This discovery underscores the importance of investigating signaling dynamics at the single-cell level. By unraveling the heterogeneity within a cell population, we gain a more comprehensive understanding of the range of responses that cells may exhibit. Single cell imaging enables the identification of subpopulations with distinct signaling profiles, revealing hidden complexities that would otherwise be overlooked in bulk measurements.

Understanding single cell heterogeneity in signaling has profound implications for both basic research and therapeutic applications [28]. It provides insights into the inherent diversity of cellular responses, offering a more accurate representation of the cellular milieu. This knowledge is crucial for designing targeted interventions, as therapies that account for and address the spectrum of cellular responses within a population are likely to be more effective. Consequently, the importance of single cell imaging in elucidating the intricacies of cellular signaling cannot be overstated. It serves as a key tool for refining our understanding of cellular behavior, fostering advancements in both basic science and clinical applications.

The convergence of eATP signaling and $[Ca^{2+}]_i$ dynamics serves as a central hub in cellular communication. Leveraging genetically encoded biosensors, particularly through multispectral imaging, provides a powerful platform for dissecting the complexities of these interconnected signaling pathways. Furthermore, the exploration of single cell heterogeneity sheds light on the diversity of cellular responses, offering a more nuanced understanding of the intricate signaling networks governing cellular behavior. Our study contributes to this ongoing endeavor by employing cutting-edge techniques to unravel the heterogeneity in eATP-induced calcium signaling, paving the way for enhanced precision and reliability in the study of cellular signaling dynamics.

Materials and Methods

Cell Culture and Transfection

Human embryonic kidney cells (HEK293T) were cultured in a high-glucose medium (4.5 g/L) supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin. Cells were maintained in a humidified incubator at 37°C with 5% CO_2. 24 hours prior to transfection, cells were seeded at a density of approximately 3×10^5 cells per well on 30 mm glass coverslips No.1 (Glaswarenfabrik Karl Knecht, Sondheim, Germany). When cells reached a confluency of about 70–80%, transfection with $GRAB_{ATP1.0}$-P2A-RCaMP was carried out using the PolyJet transfection reagent, following the manufacturer's guidelines. All imaging experiments took place 24 hours post-transfection.

Imaging Buffers and Solutions

All chemicals were purchased from NeoFroxx except otherwise stated. One hour before the imaging experiment, the culture medium was substituted with a sterile storage buffer (pH=7.43). This buffer comprised 2 mM $CaCl_2$, 5 mM KCl, 138 mM NaCl, 1 mM $MgCl_2$, 1 mM HEPES (Pan-Biotech, Aidenbach, Germany), 0.44 mM KH_2PO_4, 2.6 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 10 mM D-Glucose, 0.1% MEM Vitamins (Pan-Biotech, Aidenbach, Germany), 0.2% essential amino acids (Pan-Biotech, Aidenbach, Germany), 100 µg/mL penicillin (Pan-Biotech, Aidenbach, Germany), and 100 U/mL streptomycin (Pan-Biotech, Aidenbach, Germany). Cells were kept in the dark outside the incubator for one hour in the storage buffer before performing the imaging experiments. The physiological imaging buffer composition included 2 mM $CaCl_2$, 5 mM KCl, 138 mM NaCl, 1 mM $MgCl_2$, and 10 mM D-Glucose, pH buffered with 10 mM HEPES (7.43). For experiments with ATP administration, the desired concentrations of ATP were prepared in the abovementioned imaging buffer.

Fluorescence Imaging

Real-time imaging experiments utilizing HEK293T cells expressing $GRAB_{ATP1.0}$-P2A-RCaMP were conducted on a Zeiss Axio Observer.Z1/7 (Carl Zeiss AG, Oberkochen, Germany) equipped with a Colibri 7 LED light source (excitation wavelengths: 423/44 nm, 469/38 nm, 555/30 nm), Plan-Apochromat 20×/0.8 dry objective, Plan-Apochromat 40×/1.4 oil immersion objective, and a monochrome CCD camera Axiocam 503. $GRAB_{ATP1.0}$ signals were obtained using a 495 nm excitation filter, and

emission signals were collected through a bandpass filter (BP 525/50). For RCaMP emissions, filter combinations FT570 (BS) and emission filter 605/70 were employed. The data acquisition process utilized Zen Blue 3.1 Pro software (Carl Zeiss AG, Oberkochen, Germany). The administration and washout of the imaging buffers were facilitated by a custom-made perfusion system and a metal perfusion chamber (NGFI, Graz, Austria).

Statistical Analysis

All data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). One-way ANOVA (Tukey's multiple comparison test) was performed for statistical comparison of the multiple responses.

Results and Discussion

In this study, our primary objective was to establish a correlation between extracellular ATP levels and intracellular calcium responses within cultured HEK293T cells. To achieve this, we employed a novel bicistronic construct that co-expresses the differentially targeted $GRAB_{ATP1.0}$ for extracellular ATP detection and the intracellularly targeted calcium sensor RCaMP (Figure 1A). The distinct spectral properties of both biosensors enabled accurate detection of their emissions in their respective channels. Successful differential targeting was confirmed, and no discernible bleedthrough was observed, as illustrated in (Figure 1B). Notably, despite being separated by a self-cleavage peptide (P2A), both biosensors exhibited robust expression in the cellular environment.

Subsequently, we assessed the functionality of the biosensors and their responses to extracellular ATP, focusing on the impact on intracellular calcium levels. (Figure 1C) demonstrates that the constitutive addition of varying concentrations of extracellular ATP led to a clear and concentration-dependent activation of $GRAB_{ATP1.0}$, validating the correct function of the extracellular ATP sensor in the green channel. Concurrently, the red-fluorescent protein-based RCaMP exhibited functional responsiveness, demonstrating a robust intracellular calcium response even in the presence of low extracellular ATP concentrations.

This experimental setup, performed in transiently transfected HEK293T cells, effectively showcases the full functionality of both probes and allows for the precise correlation of extracellular ATP and intracellular calcium signals. Utilizing a pump-driven super fusion system, we administered and withdrew different concentrations of ATP, as indicated by lines. Intriguingly, we observed rapid activation of intracellular calcium, characterized by characteristic oscillations during ATP treatment. Noteworthy is the observation that upon withdrawal of the agonist from the imaging medium, extracellular ATP signals declined more slowly compared to the corresponding intracellular calcium signals.

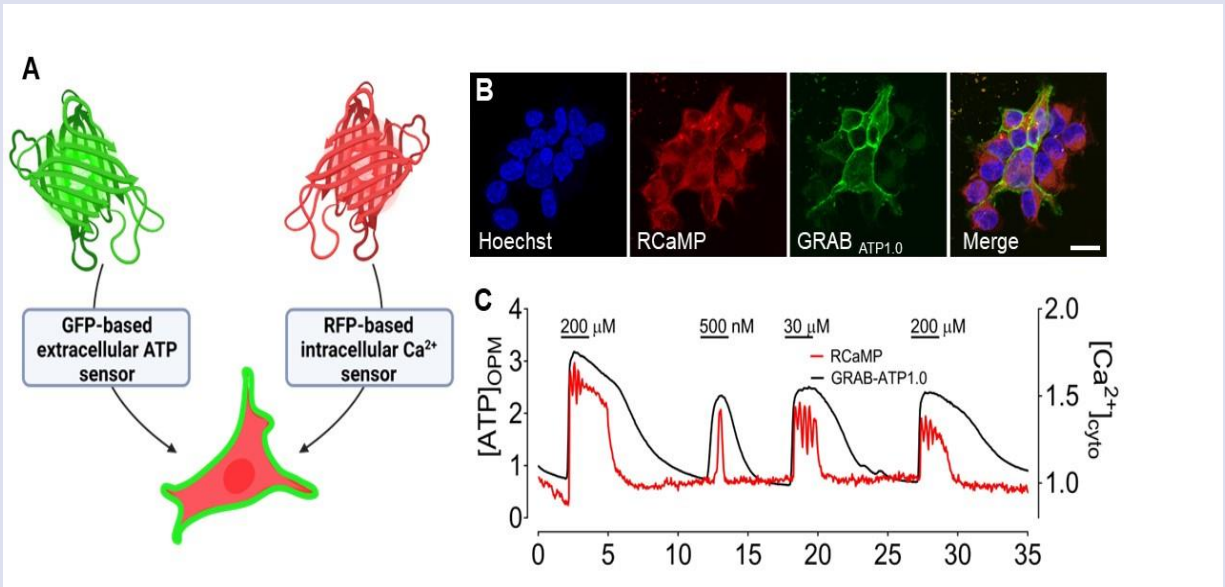

Figure 1. Graphical overview and characterization of $GRAB_{ATP1.0}$-P2A-RCaMP construct. A) Graphic illustrates the localization of GFP-based ATP biosensor ($GRAB_{ATP1.0}$) and RFP-based Ca^{2+} biosensor (RCaMP). B) High resolution confocal images of HEK293T cells expressing $GRAB_{ATP1.0}$-P2A-RCaMP. Left image shows HEK293T cells stained with Hoechst. The second and third image shows the same cells co-expressing RCamP and $GRAB_{ATP1.0}$, respectively. The very right image shows the merged version of the single images. C) Real-time traces of extracellular ATP imaged with $GRAB_{ATP1.0}$ (black curve) and intracellular calcium, imaged with RCaMP (red curve) in response to the indicated concentrations of extracellular ATP using a pump driven super fusion system. Scale bar represents 20 µm.

To further explore the observed phenomena and elucidate the direct relationship between extracellular ATP and intracellular calcium, we refined our perfusion protocols. In this iteration, we administered identical concentrations of extracellular ATP, as illustrated in (Figure 1), but omitted washout steps between the increasing concentrations of ATP. As anticipated, the extracellular ATP biosensor, $GRAB_{ATP1.0}$, exhibited a

pronounced concentration-dependent response in accordance with the increasing levels of ATP administration. Surprisingly, the corresponding intracellular calcium responses displayed non-specific calcium activity.

The observed correlation between extracellular ATP and intracellular calcium responses, as depicted in (Figure 1C), presents an intriguing aspect of cellular signaling dynamics. Notably, this correlation becomes more pronounced when extracellular ATP is subjected to a washout-readdition protocol. A plausible explanation for this phenomenon lies in the concept of receptor desensitization, a complex regulatory mechanism that shapes cellular responses to repeated or sustained ligand exposure [30]. Purinergic receptors, particularly P2 receptors that respond to extracellular ATP, are known to undergo desensitization following prolonged or repetitive stimulation [31]. Desensitization refers to a reduction in receptor responsiveness, often attributed to mechanisms such as receptor internalization, uncoupling from downstream signaling pathways, or alterations in receptor conformation. In the context of our study, repeated exposure to extracellular ATP could induce desensitization of purinergic receptors within the cellular membrane. The washout and subsequent re-addition of ATP in our experimental setup may serve as a resetting mechanism for the desensitized receptors. During the washout phase, any bound ATP molecules are removed, allowing receptors to undergo deactivation and potentially regain sensitivity. Upon re-addition of ATP, receptors that have been reset during the washout phase may exhibit heightened responsiveness to extracellular ATP, leading to the observed correlation with intracellular calcium responses. This phenomenon aligns with the concept of ligand-induced receptor desensitization and resensitization, which has been well-documented in various cellular systems [32]. It reflects the dynamic nature of cellular signaling regulation, where the responsiveness of receptors can be modulated in response to ligand exposure patterns. The washout-readdition protocol in our study provides a controlled experimental context to investigate the temporal aspects of purinergic receptor desensitization and recovery, shedding light on the intricacies of extracellular ATP-mediated signaling events.

(Figure 2) presents three representative multispectral imaging experiments (Figure 2A-C) out of several repeats. In these experiments, while certain cells exhibited minimal and transient intracellular calcium activity, others displayed oscillations irrespective of the extracellular ATP concentrations applied. The statistical analysis of these experiments, as depicted in Figure 2D, revealed a robust and heterogeneous maximum intracellular calcium response. Conversely, the extracellular ATP sensor $GRAB_{ATP1.0}$ exhibited a clear concentration correlation with no observed heterogeneity.

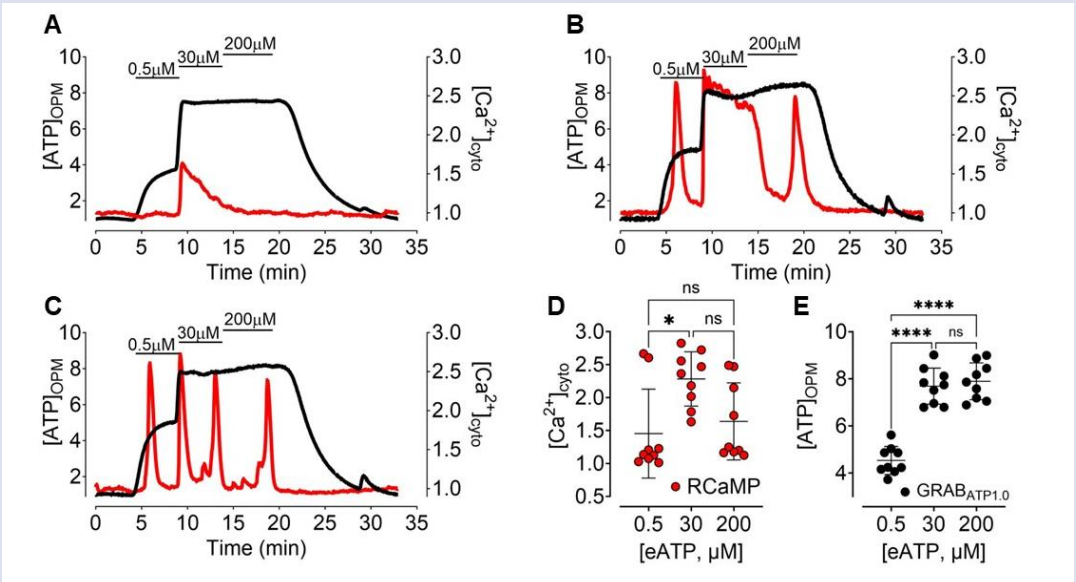

Figure 2. Single cell responses of HEK293T cells to extracellular ATP as indicated in the panels A-C. Extracellular ATP responses detected with $GRAB_{ATP1.0}$ are shown in black curve, left axis; and intracellular calcium responses detected with RCaMP are shown as red curves, right axis. These representative experiments were repeated 9 times. Panel D, shows a scatter dot plot analysis of maximum RCaMP responses (n=9) and panel e shows the statistical analysis of maximum responses of $GRAB_{ATP1.0}$ (n=9) in response to 0,5 µM, 30 µM and 200 µM as indicated in the panels. Error bars show ±SD values..

These findings collectively suggest a significant heterogeneity in intracellular calcium responses within cultured HEK293T cells exposed to identical concentrations of extracellularly applied ATP. The absence of a clear correlation between extracellular ATP and intracellular calcium responses under direct administration suggests a complex and dynamic cellular response. This lack of correlation could be attributed to the rapid and sustained activation of receptors, leading to desensitization and downstream signaling alterations

[30]. The direct administration protocol may induce receptor desensitization, disrupting the usual correlation observed with washout-readdition cycles. Receptor desensitization is a crucial aspect to consider in the interpretation of our results. P2 purinergic receptors, which respond to extracellular ATP, may undergo desensitization upon prolonged or repetitive stimulation. This desensitization could alter intracellular calcium signaling dynamics and contribute to the observed differences in responses between the two experimental setups. This unexpected diversity in the intracellular calcium signaling underscores the complexity of the cellular response to extracellular ATP and prompts further investigation into the underlying mechanisms governing such heterogeneity even in clonal cell lines.

The integration of multispectral imaging techniques has been a key facet of our research endeavors, extending beyond the investigation of extracellular ATP and intracellular calcium dynamics. In previous studies, we applied similar multispectral imaging approaches to correlate intracellular calcium responses with nitric oxide [7] or hydrogen peroxide levels [29]. In a recent investigation, we employed a multiparametric imaging strategy, revealing differential cellular responses at the subcellular level in co-cultured cells [21]. The application of multispectral imaging to simultaneously monitor extracellular ATP and intracellular calcium responses furnishes a significant advantage in our research. This methodological approach not only facilitates the detection of subtle differences in cellular responses but also allows for a nuanced exploration of intricate signaling pathways that may evade detection in conventional single-channel imaging. Our study on the interplay between extracellular ATP and intracellular calcium in HEK293T cells underscores the imperative of employing advanced imaging techniques for a comprehensive understanding of cellular signaling dynamics. Furthermore, our broader research context involving intracellular calcium, nitric oxide, and hydrogen peroxide emphasizes the versatility of multispectral imaging. By expanding our investigative scope, we have successfully unraveled the complex interrelationships governing cellular responses. In a recent multiparametric imaging study, the differential cellular responses observed in co-cultured cells on the subcellular level underscore the power of multispectral imaging in deciphering intricate cellular signaling networks [21].

The application of biosensors in single-cell imaging is paramount in uncovering cellular heterogeneity. Our study emphasizes the significance of biosensors, such as $GRAB_{ATP1.0}$ and RCaMP, in elucidating individual cell responses. Single-cell imaging allows the identification of cell-to-cell variations that would be masked in population-based measurements, providing a more nuanced understanding of cellular signaling dynamics. The detection of heterogeneous intracellular calcium responses underscores the complexity within the cellular population. Understanding this heterogeneity is crucial for several reasons. Firstly, it reflects the diverse nature of cellular responses to extracellular ATP, suggesting the involvement of intricate regulatory mechanisms. Secondly, this information has implications for therapeutic interventions, as targeting specific subpopulations of cells may be necessary for effective treatment strategies.

Conclusion

In conclusion, our study leverages advanced imaging techniques and biosensors to uncover the intricate relationship between extracellular ATP and intracellular calcium in HEK293T cells. The observed heterogeneity and the impact of receptor desensitization underscore the dynamic nature of cellular responses, emphasizing the need for nuanced experimental designs and single-cell imaging approaches in unraveling the complexity of purinergic signaling.

Acknowledgments

This research was supported by funds from the European Molecular Biology Organization EMBO (IG-5322-2023. The author acknowledges Asal Ghaffari Zaki for English language proofreading. Figure 1A was generated with Biorender (License code: ME26EKR3WY).

Conflicts of interest

There are no conflicts of interest in this work.

References

[1] Arcuino G, Lin JHC, Takano T, Liu C, Jiang L, Gao Q, Kang J, Nedergaard M. Intercellular calcium signaling mediated by point-source burst release of ATP, *Proc. Natl. Acad. Sci.*, 99 (15) (2002) 9840–9845.

[2] Cisneros-Mejorado A, Pérez-Samartín A, Gottlieb M, Matute C. ATP signaling in brain: release, excitotoxicity and potential therapeutic targets, *Cell. Mol. Neurobiol.*, 35 (1) (2015) 1–6.

[3] Depaoli MR, Karsten F, Madreiter-Sokolowski CT, Klec C, Gottschalk B, Bischof H, Eroglu E, Waldeck-Weiermair M, Simmen T, Graier WF, Malli R. Real-Time Imaging of Mitochondrial ATP Dynamics Reveals the Metabolic Setting of Single Cells, *Cell Rep.*, 25 (2) (2018) 501-512.

[4] Bonora M, Patergnani S, Rimessi A, De Marchi E, Suski JM, Bononi A, Giorgi C, Marchi S, Missiroli S, Poletti F, Wieckowski MR, Pinton P., ATP synthesis and storage, *Purinergic Signal.*, 8 (3) (2012) 343–357.

[5] Eersapah V, Hugel S, Schlichter R., High-resolution detection of ATP release from single cultured mouse dorsal horn spinal cord glial cells and its modulation by noradrenaline, *Purinergic Signal.*, 15 (3) (2019) 403–420.

[6] Fields RD, Burnstock G., Purinergic signalling in neuron-glia interactions, *Nat. Rev. Neurosci.*, 7 (6) (2006) 423–436.

[7] Eroglu E, Saravi SSS, Sorrentino A, Steinhorn B, Michel T., Discordance between eNOS phosphorylation and activation revealed by multispectral imaging and chemogenetic methods, *Proc. Natl. Acad. Sci. U. S. A.*, 116 (40) (2019) 20210–20217.

[8] Eroglu E, Hallström S, Bischof H, Opelt M, Schmidt K, Mayer B, Waldeck-Weiermair M, Graier WF, Malli R., Real-time visualization of distinct nitric oxide generation of nitric oxide synthase isoforms in single cells, *Nitric Oxide Biol. Chem.*, 70 (2017) 59–67.

[9] Barry VA, Cheek TR., Extracellular ATP triggers two functionally distinct calcium signalling pathways in PC12 cells, *J. Cell Sci.*, 107 (Pt 2) (1994) 451–462.

[10] Burnstock G., Purine and purinergic receptors, *Brain Neurosci. Adv.*, 2 (2018) 2398212818817494.

[11] Molcak H, Jiang K, Campbell CJ, Matsubara JA., Purinergic signaling via P2X receptors and mechanisms of unregulated ATP release in the outer retina and age-related macular degeneration, *Front. Neurosci.*, 17 (2023) 1216489.

[12] Eroglu E, Gottschalk B, Charoensin S, Blass S, Bischof H, Rost R, Madreiter-Sokolowski CT, Pelzmann B, Bernhart E, Sattler W, Hallström S, Malinski T, Waldeck-Weiermair M, Graier WF, Malli R., Development of novel FP-based probes for live-cell imaging of nitric oxide dynamics, *Nat. Commun.*, 7 (1) (2016).

[13] Eroglu E, Rost R, Bischof H, Blass S, Schreilechner A, Gottschalk B, Depaoli MR, Klec C, Charoensin S, Madreiter-Sokolowski CT, Ramadani J, Waldeck-Weiermair M, Graier WF, Malli R., Application of Genetically Encoded Fluorescent Nitric Oxide (NO•) Probes, the geNOps, for Real-time Imaging of NO• Signals in Single Cells, *J. Vis. Exp. JoVE*, (121) (2017) 55486.

[14] Eroglu E, Charoensin S, Bischof H, Ramadani J, Gottschalk B, Depaoli MR, Waldeck-Weiermair M, Graier WF, Malli R., Genetic biosensors for imaging nitric oxide in single cells, *Free Radic. Biol. Med.*, 128 (2018) 50–58.

[15] Wu Z, He K, Chen Y, Li H, Pan S, Li B, Liu T, Xi F, Deng F, Wang H, Du J, Jing M, Li Y., A sensitive GRAB sensor for detecting extracellular ATP in vitro and in vivo, *Neuron*, 110 (5) (2022) 770-782.

[16] Conley JM, Radhakrishnan S, Valentino SA, Tantama M., Imaging extracellular ATP with a genetically-encoded, ratiometric fluorescent sensor, *PLoS ONE*, 12 (11) (2017) e0187481.

[17] Lobas MA, Tao R, Nagai J, Kronschläger MT, Borden PM, Marvin JS, Looger LL, Khakh BS., A genetically encoded single-wavelength sensor for imaging cytosolic and cell surface ATP, *Nat. Commun.*, 10 (1) (2019) 711.

[18] Depaoli MR, Bischof H, Eroglu E, Burgstaller S, Ramadani-Muja J, Rauter T, Schinagl M, Waldeck-Weiermair M, Hay JC, Graier WF, Malli R., Live cell imaging of signaling and metabolic activities, *Pharmacol. Ther.*, 202 (2019) 98–119.

[19] Li ES, Saha MS., Optimizing Calcium Detection Methods in Animal Systems: A Sandbox for Synthetic Biology, *Biomolecules*, 11 (3) (2021) 343.

[20] Ghaffari Zaki A, Yiğit EN, Aydın MŞ, Vatandaslar E, Öztürk G, Eroglu E., Genetically Encoded Biosensors Unveil Neuronal Injury Dynamics via Multichromatic ATP and Calcium Imaging, *ACS Sens.*, 9 (3) (2024) 1261-1271.

[21] Secilmis M, Altun HY, Pilic J, Erdogan YC, Cokluk Z, Ata BN, Sevimli G, Zaki AG, Yigit EN, Öztürk G, Malli R, Eroglu E., A Co-Culture-Based Multiparametric Imaging Technique to Dissect Local H2O2 Signals with Targeted HyPer7, *Biosensors*, 11 (9) (2021) 338.

[22] Akerboom J, Carreras Calderón N, Tian L, Wabnig S, Prigge M, Tolö J, Gordus A, Orger M, Severi K, Macklin J, Patel R, Pulver S, Wardill T, Fischer E, Schüler C, Chen TW, Sarkisyan K, Marvin J, Bargmann C, Kim D, Kügler S, Lagnado L, Hegemann P, Gottschalk A, Schreiter E, Looger L., Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics, *Front. Mol. Neurosci.*, 6 (2013) Available at: https://www.frontiersin.org/articles/10.3389/fnmol.2013.00002. Retrieved Oct. 23, 2022.

[23] Arai S, Kriszt R, Harada K, Looi LS, Matsuda S, Wongso D, Suo S, Ishiura S, Tseng YH, Raghunath M, Ito T, Tsuboi T, Kitaguchi T., RGB-color intensiometric indicators visualize spatiotemporal dynamics of ATP in single cells, *Angew. Chem. Int. Ed Engl.*, 57 (34) (2018) 10873–10878.

[24] Gupta RK, Kuznicki J., Biological and Medical Importance of Cellular Heterogeneity Deciphered by Single-Cell RNA Sequencing, *Cells*, 9 (8) (2020) 1751.

[25] Hastings JF, Latham SL, Kamili A, Wheatley MS, Han JZR, Wong-Erasmus M, Phimmachanh M, Nobis M, Pantarelli C, Cadell AL, O'Donnell YEI, Leong KH, Lynn S, Geng FS, Cui L, Yan S, Achinger-Kawecka J, Stirzaker C, Norris MD, Haber M, Trahair TN, Speleman F, De Preter K, Cowley MJ, Bogdanovic O, Timpson P, Cox TR, Kolch W, Fletcher JI, Fey D, Croucher DR., Memory of stochastic single-cell apoptotic signaling promotes chemoresistance in neuroblastoma, *Sci. Adv.*, 9 (9) eabp8314.

[26] Handly LN, Yao J, Wollman R., Signal transduction at the single-cell level: Approaches to study the dynamic nature of signaling networks, *J. Mol. Biol.*, 428 (19) (2016) 3669.

[27] Mattiazzi Usaj M, Yeung CHL, Friesen H, Boone C, Andrews BJ., Single-cell image analysis to explore cell-to-cell heterogeneity in isogenic populations, *Cell Syst.*, 12 (6) (2021) 608–621.

[28] Baysoy A, Bai Z, Satija R, Fan R., The technological landscape and applications of single-cell multi-omics, *Nat. Rev. Mol. Cell Biol.*, 24 (10) (2023) 695–713.

[29] Altun HY, Secilmis M, Caglar TA, Vatandaslar E, Öztürk G, Vilain S, Eroglu E., Visualizing H2O2 and NO in endothelial cells: strategies and pitfalls. *bioRxiv*, Available at: https://www.biorxiv.org/content/10.1101/2023.02.15.528776v1. Retrieved Feb. 16, 2023.

[30] Shankaran H, Wiley HS, Resat H., Receptor downregulation and desensitization enhance the information processing ability of signalling receptors, *BMC Syst. Biol.*, 1 (2007) 48.

[31] Rodríguez-Rodríguez R, Yarova P, Winter P, Dora K., Desensitization of endothelial P2Y1 receptors by PKC-dependent mechanisms in pressurized rat small mesenteric arteries, *Br. J. Pharmacol.*, 158 (6) (2009) 1609–1620.

[32] Woolf PJ, Linderman JJ., Untangling Ligand Induced Activation and Desensitization of G-Protein–Coupled Receptors, *Biophys. J.*, 84 (1) (2003) 3–13.