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Bioluminescence imaging on-chip platforms for non-invasive high-content bioimaging

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1. Introduction

Current in vitro methods for drug screening are mostly based on 2D monolayer cell culture models, failing to depict an accurate representation of normal cell behavior on the in vivo environment (Edmondson et al., 2014). Specifically, although 2D cultures are considered cost-effective and easy-to-image strategy to study cell responses, these systems fail in recapitulating complex 3D microenvironments, including cell-cell, cell-matrix, and intra-/inter-organ interactions (Chen et al., 2021; Fang and Eglen, 2017). Altogether, conventional in vitro outcomes based on these models largely fail to mimic and predict complex cell behavior.

Moreover, animal models can only partly recapitulate human physiological responses due to the discrepancies between the distinct biological systems (Akhtar, 2015). In addition, the progressive increase in ethical concerns over animal welfare (e.g. 3R) contributes to the accelerated development of alternatives with better predictive outcomes for drug development and screening (Accomasso et al., 2018).

The development of microfluidic-based microphysiological systems, also named organs-on-chips (OoC), represents a promising alternative to conventional in vitro models, due to their accurate mimicry of in vivo settings. In short, OoC offer the unique possibility of providing key

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ABSTRACT

Incorporating non-invasive biosensing features in organ-on-chip models is of paramount importance for a wider implementation of these advanced in vitro microfluidic platforms. Optical biosensors, based on Bioluminescence Imaging (BLI), enable continuous, non-invasive, and in-situ imaging of cells, tissues or miniaturized organs without the drawbacks of conventional fluorescence imaging. Here, we report the first-of-its-kind integration and optimization of BLI in microfluidic chips, for non-invasive imaging of multiple biological readouts. The cell line HEK293T-GFP was engineered to express NanoLuc® luciferase under the control of a constitutive promoter and were cultured on-chip in 3D, in standard ECM-like hydrogels, to assess optimal cell detection conditions. Using real-time in-vitro dual-color microscopy, Bioluminescence (BL) and fluorescence (FL) were detectable using distinct imaging setups. Detection of the bioluminescent signals were observed at single cell resolution on-chip 20 min post-addition of Furimazine substrate and under perfusion. All hydrogels enabled BLI with higher signal-to-noise ratios as compared to fluorescence. For instance, agarose gels showed a ~5-fold greater BL signal over background after injection of the substrate as compared to the FL signal. The use of BL with microfluidic chip technologies opens up the potential for simultaneous in situ detection with continuous monitoring of multicolor cell reporters. Moreover, this can be achieved in a non-invasive manner. BL has great promise as a highly desirable biosensor for studying organ-on-chip platforms.
biochemical, biomechanical, and biocompatible cues to faithfully mimic cell, tissue, and/or organ functions in health and disease (Leung et al., 2022). Thus, this technology enables recreation of organ-like physiology, as well as pathophysiological tissue responses of various tissues and organs, such as the brain-blood barrier (Kim et al., 2023; Park et al., 2019), lung (Baptista et al., 2022; Sisodia et al., 2022), gut (Verhuls et al., 2021), and cartilage (Occhetta et al., 2019; Paggi et al. 2020, 2022).

Apart from the cost-effective and patient-specific features of these complex engineered 3D models, the integrative nature of organ-on-chip with other technologies, offers unexplored and promising pathways for non-invasive and real-time monitoring, and detection of physiological behavior with single-cell resolution (Fuchs et al., 2021). Specifically, integration of electrochemical and optical technologies increases the versatility of organs-on-chip for monitoring at a sub-micron to micron resolution. These on-chip imaging technologies are enticing, with a wide range of potential applications, such as non-invasive biomedical imaging and implementation on automated and miniaturized systems (Takehara et al., 2017).

To date, fluorescence detection is the standard optical biosensing method used for cell analysis on-chip (Song et al., 2019; Vargas-Ordaz et al., 2021). Real-time fluorescence cell imaging and biomarker detection typically requires the need of end-point analysis, which is invasive and/or terminal, often involving complete sample sacrifice (Cullum et al., 2014; Wlodkowic et al., 2009). Moreover, cell bleaching can potentially occur due to laser exposure, compromising continuous monitoring of cell status, besides possibly detrimentally affecting cell behavior, thereby biasing the derived data representativeness (Laisse et al., 2017).

In this work, a bioluminescence imaging (BLI) approach was combined with a standard OoC platform. BLI is becoming more prominent due to its compatibility with real-time, sensitive monitoring of molecular and cellular processes (Prescher and Contag, 2010). Although the fluorescence emission can be significantly higher than bioluminescence (BL), BL signals can be highly specific to a given biological process because of its exceedingly low background signal (Kim et al., 2017). BLI relies on the detection of visible light driven by luciferase-dependent oxidation of a molecular substrate when the enzyme is expressed as a molecular reporter (Sadikot and Blackwell, 2005). Therefore, the BL signal is generated without external light and the background emission is negligible. Moreover, BLI lacks phototoxicity which allows for long-term imaging of living cells, even in 3D tissues such as embryos (Moraes-Curiel et al., 2022), organoids (Santos et al., 2022) and freely moving animals (Moraes-Curiel et al., 2022).

Specifically, BLI strategies can accurately detect the presence of physiologically relevant BL proteins at extremely low concentrations (at the nM range), with no interference from other physiological fluids (Yu et al., 2019). Moreover, this technology is compatible with distinct miniaturized systems and consequently suited for high-throughput monitoring.

BLI is currently a widely regarded technique used in a variety of cell biological and pharmacological analysis. In the last years, it has been specifically optimized for noninvasive visualization of single cells in freely moving animals and also for simultaneous visualization of multicolor bioluminescent cells for in vitro deep tissue imaging in the fields of immunology and tumor research (Iwano et al., 2018; Mezzonotte et al., 2014; Schomann et al., 2020; Zambito et al., 2022). However, many suboptimal limitations have hindered its integration onto micro-physiological systems, mostly due to limited function outputs for non-invasive fully autonomous microsystems (Moreira Teixeira and Mezzonotte, 2021).

These factors are mainly related to the use of stable and soluble substrates, which have poor membrane permeability, thereby reducing the bioavailability inside tissue and in animals, which consequently lowers the quantum yield and emission of photons (Kim et al., 2022). In the last decade, novel BL systems have been developed based on the mutagenesis of natural luciferases and the synthetic optimization of luciferin substrates. The optimization of substrates and enzymes through chemical and bioengineering advances have greatly improved the photon emission versatility. For instance, NanoLuc luciferase paired with Furimazine substrate is characterized by high brightness and stability, making it widely adopted for imaging studies in diverse fields (Yao et al., 2018). Specifically, NanoLuc showed improved BL properties regarding signal intensity and stability of reaction. Recently, bioluminescence resonance energy transfer (BRET) has increased the potential applications of NanoLuc paired with a fluorophore (Nano-BRET) to monitor proximity within living cells resulting in a quantifiable bright BL system (Buchanan and Yoon, 2022; Kim and Gralke, 2016). Therefore, a whole spectrum of light-emitting proteins can be tailored to a specific need (Suzuki et al., 2021). Thus, multicolor emission and improved brightness can be achieved for visualization of multiple cellular events, which could be applied for on-chip measurements (Michelini et al., 2019; Sheyn et al., 2019).

Here, we attempted the first-of-its kind integration of BLI on an on-chip platform, with the final goal of establishing a single-cell imaging protocol for multiple and continuous biological readouts. To achieve that, we genetically engineered the HEK-293T cell line to express NanoLuc luciferase under the control of a constitutive promoter. NanoLuc is a bright and small luciferase that gives high sensitivity to BLI. BL detection is allowed with the substrate Furimazine, which is cell permeable and has strongly reduced auto-luminescence thereby decreasing the assay background. HEK293T-NanoLuc-GFP cells were integrated into three commercially available extracellular matrix-like hydrogels with distinct mechanical properties (Agarose, GelMA, and Fibrin) and cultured in a prototypic organ on chip design. First, the distinct hydrogels were screened regarding diffusion and mechanical properties to prove BLI versatility. BLI of HEK293T-NanoLuc-GFP cells was first tested using the IVIS imager where the luciferase spectrum, photon flux and luciferase/luciferin kinetics were detected and measured over time. We further performed BL single-cell imaging on-chip using an adapted confocal microscope equipped with a sensitive EMCCD camera. We achieved luminescence from living cells after dynamic infusion of the Furimazine substrate. BL at single-cell resolution was detected with multiple standard hydrogels used in OoC platforms, namely agarose, GelMA (Gelatin-Methacyrlate), and fibrin hydrogels. The results suggest that on-chip incorporation of highly sensitive bioluminescent signals improved SNR as compared to standard fluorescence detection, which supports that this may be a valuable combination of advanced tools for non-invasive in vitro detection of cell behavior.

2. Experimental section

2.1. Microfluidic chip fabrication

Micropatterned SU-8 master molds were produced. Briefly, h100i orientation silicon wafers (Okmetic, Finland) were spin-coated with an SU-8100 negative photoresist (Microchem, USA), producing an average layer height of the central chamber of ~250 μm. The SU-8 photoresist was then patterned by exposure to UV light with a 365 nm longpass filter using an EVG 620 mask aligner (EVGroup, Austria). The patterned wafers were then developed in RER600 (Fujifilm, Japan) followed by spraying, spinning, and drying. Finally, patterned wafers were washed with isopropanol (IPA) and dried using a stream of nitrogen gas. Microfluidic chip devices were produced by soft lithography using polydimethylsiloxane (PDMS). In brief, a mixture of curing agent (Sylgard 184, Dow Corning, USA) and PDMS prepolymer in a 1:10 ratio was degassed before being poured onto SU-8 molds. PDMS was then cured at 65 °C in an oven overnight. The following day, the patterned PDMS was peeled from the SU-8 wafer and cut to the defined shape. The central chamber inlets/outlets and the perfusion (media) inlets/outlets were punched with 1.5 and 1 mm Ø biopsy punches, respectively.
2.2. Hydrogel preparation and diffusion study

Three commercially available hydrogels were used to test the compatibility of BLI with several materials commonly used for cell encapsulation on organ-on-chip devices, namely agarose, GelMA, and fibrin.

Agarose was prepared by dissolving low-melting agarose powder (UltraPure Agarose, Invitrogen, United States) in 1×PBS to a final concentration of 2% (w/v). The solution was heated in a microwave until agarose was completely dissolved in the saline and placed on a water bath (∼55 °C) to avoid jellification until injection on-chip. The agarose hydrogel solution was then injected in the central chamber of the chip and incubated at room temperature for 5 min to achieve complete jellification.

GelMA (Gelatin Methacryloyloyl) (Sigma-Aldrich, United States) hydrogel was fabricated by mixing GelMA with the photoinitiator LAP (Lithium phenyl-2,4,6-trimethylbenzoylphosphinate, Sigma-Aldrich, United-States) dissolved in 1×PBS to a final concentration of 4% (w/v). Prior to UV crosslinking, the hydrogel solution was injected into the central chamber of the chip. The hydrogel-containing chip was then exposed to UV light for 1–2 min to obtain complete crosslinking of the GelMA hydrogel.

Fibrin gel was fabricated by mixing ELAREM™ Perform I GMP Grade platelet lysate (PL Bioscience, Aachen, Germany) with a thrombin from bovine plasma (Sigma-Aldrich, United States) dissolved in a CaCl₂ (concentration of this solution in molar) solution to a final concentration of 10 U thrombin per ml of 0.2M CaCl₂ solution to a final concentration of 2% (w/v). The solution was heated in a microwave until agarose was completely dissolved in the saline and placed on a water bath (∼55 °C) to avoid jellification until injection on-chip. The agarose hydrogel solution was then injected in the central chamber of the chip and incubated at room temperature for 5 min to achieve complete jellification.

2.3. Mechanical characterization of hydrogels

Polymer precursor solutions were casted on top of a 6 mm diameter PDMS ring with 1 mm height to obtain a homogeneous hydrogel post crosslinking. Young’s moduli were determined using interferometry-based nanoindentation (Optics11, Pavone) using a cantilever (spring constant 0.27 Nm⁻¹) with a glass colloidal probe (radius = 11 μm) attached to the tip. All measurements were performed with samples immersed in PBS. Indentation depth was chosen based on the tip radius and thickness of the sample. The hydrogel discs were indented to 1000 nm for 2 s followed by a 1 s holding and 2 s retraction time. The obtained indentation curves were fitted using the Hertzian model with Pmax value of 70% from which the elastic modulus was obtained using the formula E=Eeff(1−ν²), where E is the Young’s modulus, Eeff effective Young’s modulus with assuming a Poisson’s ratio of ν=0.5. The samples were further characterized for nano topographies by performing a 3x3 matrix scan for each sample, and Young’s modulus was plotted over an area of 90 μm² for each condition. Supplementary data

2.4. Cell culture

Human embryonic kidney (HEK-293T) cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured in T-175 flasks (Thermo Fisher Scientific) until reaching confluence; growth media was aspirated, and cells were washed with PBS (Sigma-Aldrich). The PBS was aspirated, and the cells were removed from the flask by the addition of 3 mL of TrypLE™ Express Trypsin (Life Technologies) and were incubated at 37 °C. Cells were centrifuged at 1100 RPM, washed, re-suspended in 5 mL of fresh growth media, and then counted using a BioRad TC20 cell counter. The culture was incubated at 37 °C with 5% CO₂. Briefly, the tested hydrogel precursors were prepared as described above and cells were included on the hydrogel precursor solution following trypsinization with 0.25% Trypsin-EDTA.

2.5. Lentiviral production

The cDNA of Nanoluc was provided by Promega (ID: #137777 on Addgene) in a pCDH-EF1-T2A-copGFP lentiviral vector for expression in mammalian cell. Viral production and cell transduction were performed under appropriate biosafety level conditions (ML-II) in accordance with the National Biosafety Guidelines and Regulations for Research on Genetically Modified Organisms. Procedures and protocols were reviewed and approved by the EMC Biosafety Committee (GMO permit 99–163). Lentiviral particles were produced by transfection of HEK-293T packaging cells with three packaging plasmids (pCMV-VSVG, pMDLg-RRE (gag-pol), pRSV-REV (Addgene) and the lentiviral vector plasmid using PEI transfection reagent (1 mg/mL)/μg DNA. The supernatant, containing lentiviral particles, was collected after 48 h and 72 h. Viral quantification was performed using the standard antigen-capture HIV p24 ELISA (ZeptoMetrix).

2.6. Generation of luciferase-expressing HEK-293T cell lines

HEK-293T cells were grown in culture dishes to 50% confluence in culture medium and were transduced with an MOI of 1 using pCDH-EF1-NanoLuc-T2A-copGFP lentivirus plus with polybreine (hexamethrime bromide) (Sigma-Aldrich) at the final concentration of 8 μg/mL. Cells were sorted for GFP expression (excitation/emission maximum = 475/509 nm) using FACS (BD-FACS AriaIII, BD Biosciences). Stably transduced cells (HEK293T-NanoLuc-GFP) were expanded and used in subsequent experiments.

2.7. In vitro cell-based imaging by the IVIS spectrum imager

HEK-293T-NanoLuc cells were seeded in the microfluidic chip at a density of 2,5x10⁴ cells per chamber. Post 24 h, cells were imaged at the IVIS Spectrum Imager (PerkinElmer) after addition of Furimazine substrate in a final concentration of 20 μM. The following setting was applied: FOV C, f/stop = 1, medium binning, 1 s exposure time, and a range of bandpass filters ranging from 500 nm to 600 nm. For kinetics analysis the bioluminescence signal was measured every 2 min after substrate addition with 1 s acquisition time with an open filter.
Experiments were performed in triplicates and were repeated three times. Data was analyzed using Living Image 4.3 software (Perkin Elmer) by applying the appropriate region of interest (ROI).

2.10. In vitro cell-based bioluminescent imaging by confocal microscopy equipped with EMCCD camera

Bioluminescence was imaged in luciferase-expressing HEK293T-NanoLuc-GFP cells by adding 20 μM of Furimazine as a substrate (Promega). Because of the fast enzymatic kinetics of Nanoluc luciferase with the Furimazine substrate, cells were imaged immediately after addition of the substrate. The images were captured every 2 min for 15 min. The chips were imaged using a Zeiss Elyra PS1 SIM microscope installed with a sensitive EMCCD camera (Andor iXon DU 885, 1002 × 1004 pixels), cooled at −64 °C and with 10 × magnification lens. These settings enabled the full range of the detector’s sensitivity with exposure time of 1 s.

For chips imaged under flow, a syringe pump was used (Harvard apparatus, PHD ultra) with a flow rate of 10 μl/min. Two syringes of 1 ml volume (BD Plastipak), were placed in the pump with 500 μl of complete DMEM medium supplemented with 20 μM of Furimazine. Images were acquired every 2 min for 15 min and relative light units (RLU) were measured over time. RLU quantification was performed by selecting relevant regions of interest (ROI) within the tile scans obtained, both at the edges and center of the imaging chamber of the chip. The mean pixel intensity was found by the average of the 4 mean intensities of the distinct ROI. For the analysis of signal-to-noise ratio (SNR), images were acquired 10 min after addition of Furimazine substrate and appropriate ROIs were drawn for single cells in either the BL channel and FL channel. RLU quantifications were based on images treated using ImageJ (Fiji). SNR ratios were calculated by dividing the average of mean intensities of the signal bioluminescence minus the average mean intensities of the background divided to the SD of the mean intensity of the background. All the graphs were plotted using...

Fig. 1. Design of the microfluidic platform and assessment of the mechanical properties of the 3D environments to integrate bioluminescence. a) Top view of the chip b) schematic depicting the chip’s dimensions, depicting a cross-section and longitudinal cross-section, composed of a main central chamber and two media perfusion channels, interconnected by an array of pillars to provide nutrient exchange to the encapsulated cells. c)-e) To assess and quantify the differential diffusion times on the different hydrogels, Dextran-FITC (40 kDa) was injected through the media perfusion channels and diffusion was quantified for a period of 40 min. f) % gel fraction, g) swelling ratio and h) Young’s modulus of the tested hydrogels. (One-way ANOVA * p<0.05).
2.11. Statistical analysis

For experiments where differences between two groups were compared, ordinary one-way ANOVA followed by t-test was used. For experiments where more than two groups were compared, ordinary one-way ANOVA followed by Tukey’s test was used to determine significant differences among groups. p values < 0.05 were considered statistically significant. Bioluminescent signals were quantified by drawing ROIs with ImageJ and statistical analysis was performed in Microsoft Excel. Error bars represent ± SD.

3. Results and discussion

3.1. Characterisation of the 3D environments for BLI detection on-chip

The microfluidic chip design used for this study comprises a central chamber (Fig. 1 a)- Top) used for optical detection and culture of the cell-laden hydrogels, sided bilaterally by media perfusion channels (Fig. 1 a - Bottom). The outer dimensions of the chip are 1.7x1,2x0,4 cm (Fig. 1 b). The optical detection chamber has dimensions of 7,5x2x0,25 mm. The total volume of the central chamber of the chip is 10 μl. Prior to cell seeding on-chip, we proceeded with characterization of material properties of the selected three hydrogels. These ECM-like hydrogels have been widely used for 3D cultures (Hasenberg et al., 2015; Liu et al., 2020; Paggi et al., 2022) and were selected to establish and analyze potential differences in the compatibility with differential imaging on-chip modalities, such as fluorescence vs bioluminescent imaging. They were chosen primarily on their mechanical properties, specifically a very low stiffness hydrogel (Fibrin ~0.5 kDa), an intermediate hydrogel (GelMA ~2 kDa), and a relatively stiff hydrogel (Agarose >10 kDa), also comprising different crosslinking strategies, enzymatic, UV-induced, and temperature-mediated, respectively. First, we determined the diffusion rate between hydrogels by injection with Dex-FITC (40 kDa) or FITC (0.37 kDa) through the media perfusion channels. As BL relies on the contact of the substrate Furimazine with the bioengineered NanoLuc cells, the purpose of this experiment was to assess and confirm complete and rapid substrate diffusion times on the distinct ECM-like hydrogels. The two distinct fluorophores used are between the range of the substrate (MW 381.43 g/mol) and therefore had expected different diffusion times. As shown on Fig. 1 d), Dex-FITC (40 kDa) has diffused completely in all the hydrogels tested within the timeframe of the experiment (40 min). Due to their intrinsic and specific mechanical properties, each hydrogel had expected diffusion profiles, reaching complete diffusion within 5 min for fibrin gel, 20 min for GelMA, and 35 min for agarose (Fig. 1 e). These gels were then measured regarding percentage hydrogel fraction (Fig. 1 f), swelling ratios (Fig. 1 g), and young’s modulus (Fig. 1 h). Results show that, after 1 day incubated in PBS, the degree of crosslinking formed in the structure hydrogel polymer network (%gel fraction) is significantly (p<0.05) higher in the GelMA than in agarose. This is owed to the higher GelMA porosity over the agarose gel, consequently displaying higher swelling and % gel fraction. This was confirmed by the swelling ratio quantification as the fractional increase in the weight of the hydrogel measurements after 1 day in 1xPBS was higher for GelMA hydrogel than agarose hydrogel. The absence of fibrin gel/gel fraction and swelling ratios led to measurement difficulties due to very low gel stiffness, thus these values were not included in this work. Nonetheless, Hasanzadeh et al., (2020) have measured the properties of fibrin, with similar composition, presenting values close to zero, thereby not being able to observe any significant changes on these specific properties.

After confirming that each hydrogel allows for homogenous substrate diffusion within the chamber compartment of the chip platform, we then optimized cell seeding density to obtain single cell resolution on the bright luminescence signal. As shown on the brightfield pictures presented in Fig. 2 a, a cell density between 10x10⁶ and 2x10⁴ cells/chip is indicated to obtain single cell-resolution in the on-chip optical chamber, naturally dependent on the height of the focal point due to the 3D geometry of the central chamber/hydrogel. For the remainder of the experiments, 2x10⁴ cells/chip was adopted as the standard cell seeding density to obtain single-cell resolution. Importantly, in all of the tested gels, cell viability was assessed 24h post-seeding, with >90% of hydrogel-embedded cells on the central chamber being stained for Calcein-AM (≥ 150 cells/chip) (Fig. 2 b-c).

3.2. Bioengineered HEK-NanoLuc provide detectable and measurable spectrums of luminescence on-chip

The HEK-293T cell line was engineered for equimolar expression of NanoLuc luciferase mutant and green fluorescence protein (GFP) (HEK293T-NanoLuc-GFP). To study the feasibility of BLI on-chip, four different concentrations of HEK-NanoLuc cells ranging from 2,5x10⁵ to 25x10⁴ cells/chip, were seeded in the chip and spectrally imaged after the addition of Furimazine substrate (20 μM) on the IVIS spectrum imager at 37 °C (Fig. 3 b). Bioluminescence photon emissions were recorded post-addition of Furimazine every 1 min for 20 min. A proportional correlation between the number of cells seeded and the photon fluxes was obtained, confirming that BL derived from HEK-NanoLuc cells encapsulated in a 3D hydrogel network can be efficiently detected and quantified. (Fig. 3 b). Specifically, the emission measured as number of photons per second was the greatest (~3.6x10⁹ photons/s, 5 min post substrate injection) for the chip seeded with the highest cell density (2.5x10⁵ cells) compared to the chip seeded with 1.5x10⁵ cells (0.7-fold lower brightness) and compared to the chips seeded with 5x10⁴ and 2.5x10⁴ cells (0.55-fold lower brightness). However, the highest two concentrations resulted in saturated images that may affect the reliability of the BL recorded (data not shown). Interestingly, enzymatic activity peak was registered after 4–5 min post-substrate addition and BL outputs of the four cell densities tested stable over 15 min (Fig. 3 b). In Fig. 3 c (left panel), a representative BL image of the chip seeded with 2.5x10⁵ HEK-NanoLuc is depicted. Recorded emission peak for NanoLuc was around 500 nm instead of 460 nm [28] due to limitation of the CCD camera detection (Fig. 3 c, right panel), which has no filter in the range of 400–500 nm. By imaging and quantifying using the IVIS spectrum, we demonstrate that the emission flux of the NanoLuc/Furimazine system is high enough to be imaged by adapted microscope for BLI, for non-invasive monitoring of the cell status.

3.3. Dynamic injection into microfluidics chip allows sensitive BLI

An electron-multiplying CCD (EM-CCD) camera was used to equip a conventional confocal microscope, to yield higher sensitivity and image quality suitable for BLI microscopy, which was used to image the microfluidic chip platform, with hydrogel-encapsulated HEK293T-NanoLuc-GFP cells. To achieve multicolor-imaging, brightfield, fluorescence, and bioluminescence were recorded upon dynamic injection of the furimazine substrate.

Representative images of the tile scans of the chips seeded with HEK-NanoLuc cells encapsulated with the three types of hydrogels (Agarose, Fibrin, and GelMA), are shown in Fig. 4 a-c. The diffusion of the Furimazine substrate and the sequential bioluminescence activity from the edges to the core of the chip was observable and quantifiable over a period of 20 min, which is consistent with the spectrum observed by IVIS spectrum imager. The relative light unit (RLU) registered show activation of encapsulated cells after 20 min, which showed similar activation profiles for agarose and fibrin gels, which were different from those observed for GelMA. For agarose and fibrin gels, BL output of HEK-NanoLuc detected at the edge of the chip produced the highest signals ~1 min after substrate injection with 1.8x10⁵ RLU registered for HEK293T-NanoLuc-GFP in agarose, and 1.1x10⁵ RLU registered for HEK-NanoLuc in fibrin. Mild decrease of the
BL yields was recorded for the latter time points (5–20 min). When GelMA was used to encapsulate HEK293T-NanoLuc-GFP cells, the highest BL outputs were produced at 15 min post-substrate injection (1.7x10^4 RLU) (Fig. 4 b, left panel). In contrast, for agarose gel, a gradual increase of luminescent signal with a BLI peak at 15 min (4.4x10^3 RLU) is detected in the center of the chip, in contrast to an almost immediate and luminescent stable signal for Fibrin gel (4.2x10^3 RLU) (Fig. 4 e, right panel) throughout the duration of the experiment. Specifically, fibrin gel shows an almost immediate BLI signal activation of HEK-NanoLuc cells throughout the whole chip already after 1-min post-substrate injection. This is most likely due to its rapid diffusion profile that allows to produce a 4.8-fold higher signal output compared to agarose gel (Fig. 4 e, right panel). Finally, when GelMA was used as hydrogel, HEK-NanoLuc cells had a little-to-no BL activation in the central chamber of the chip most likely due to insufficient diffusion of the substrate to the core of the hydrogel throughout the 20 min of BL detection. These results are consistent with the expected diffusion profile of each of these gels as observed in Fig. 2. These results correlate to the mechanical properties, namely to the stiffness of the hydrogels, indicating that higher stiffness levels may compromise uniform diffusion of the substrate throughout the OoC chamber. Thus, understanding and characterizing both the mechanical properties (specially diffusion rates) of which hydrogel and which on-chip platform design to use is key to provide a detectable and clear BLI detection signal.

On par, BLI detection of more complex structures such as 3D-spheroids was proven to be feasible (Supplementary data S3), yet requiring some imaging optimization or the use of more sensitive cameras to achieve greater resolution.

The BL system NanoLuc/Furimazine exploited here, revealed a high bioluminescence intensity but a limited number of photons that required the maximum exposure time allowed by the adapted microscope (1 s). To circumvent this issue some solutions can be adopted such as using a new chemical substrate such as Hydrofurimazine (HFz) and Fluorofurimazine (FFz), which are more soluble, enable high sensitivity and prolonged light production (Su et al., 2020).

Recently, Morales-Curiel et al. showed that by using bioluminescence as imaging modality for fast cellular dynamics with a deep learning model, it’s possible to increase the SNR (Morales-Curiel et al., 2022). Thus, by overcoming several challenges, they demonstrated the use of bioluminescence as an imaging modality that could also be applied in the millisecond range.

3.4. Bioluminescence on-chip provides favorable signal-to-noise ratios compared to fluorescence

The use of various fluorescent reporters as an optical approach for
live cell imaging on 2D settings offers versatile possibilities, due to the broad spectrum of wavelengths that can be integrated with significant spatial and temporal resolution (Tung et al., 2016; Welsh and Kay, 2005). This usually relies on the use of light sources, typically high-intensity lasers, to excite fluorescent molecules which, in turn, emit specific wavelengths. Although the integration of fluorescent approaches for live cell imaging for 3D substrates is already established and has become the gold standard as an optical detection method for on-chip devices, critical drawbacks remain, limiting and hampering the progress of the OoC field. In particular, imaging depth, spatial resolution, temporal resolution, and photo-damage are some of the drawbacks that are in urgent need to be solved with novel imaging approaches (Chin et al., 2016). The integration of BLI for live cell imaging on 3D substrates such as on-chip platforms could potentially circumvent these
To investigate if the BLI signals captured by the EMCCD camera could generate a high SNR intensity over conventional fluorescence imaging, we registered the BL emission of HEK293T-NanoLuc-GFP cells encapsulated with agarose, fibrin, and GelMA on-chip. Thus, upon injection of the Furimazine substrate and subsequent NanoLuc luciferase activation, single cells showed a consistent and significant BL output for all three hydrogels (Fig. 5). Amongst the three hydrogels, agarose and fibrin gels showed approximately a 1.6-fold higher BL SNR over FL at 10 min after injection of the substrate (Fig. 5 a-a’; b-b’). Instead GelMA possessed a 5-fold higher BL SNR over FL. Thus, the BL signal does not appear to be directly correlated to the stiffness of the hydrogels yet may be related to their translucency degree. This fact highlights the significant and effective capture of high-intensity photons on-chip through bioluminescent imaging on a 3D substrate such as hydrogels embedded in OoC platforms. In line with this, the results obtained also show the FLI BLU ratios observed are largely impacted by the existence of autofluorescence on the central chamber of the chip, on all the tested hydrogels. PDMS-based chips are known to produce some autofluorescence on the central chamber of the chip, for instance, the use of Nano-lanterns developed from the wild type Renilla reniformis luciferase coupled with yellow, cyan, or orange luminescent proteins enable multicolor imaging based on BRET. The brightness achieved was approximately 20 times brighter than the wild-type Renilla luciferase. This allowed simultaneous monitoring of the expression of multiple genes with high spatial and temporal resolution (Takai et al., 2015). Therefore, we envision a promising application for the visualization of two or more biological events encapsulated in the same microfluidic chip.

A recent study reported that furimazine possibly displays toxic side effects, in which the authors observe and conclude that its toxicity is individual and dependent on the cell line used (Shipunova et al., 2018). Although we acknowledge that furimazine might present an effect on cell viability (in this case, cell death), we have not observed a significant toxicity of this compound at 20 μM (Supplementary Figs. S4–A). Moreover, Yao Z. et al. demonstrated that higher concentrations of furimazine (25–50 μM) have been used on other bioluminescence imaging studies where continuous, excitation-free imaging of tumor spheroids was performed continuously with no observable impact on cell viability (Yao et al., 2022). However, we cannot rule out other concentration-dependent effects for specific cell types (e.g. growth inhibition, DNA damage).

Overall, the system we developed could be ideally suited to monitor living patient-derived cells and assess cell cytotoxicity or tissue formation progress, non-invasively and continuously over time. Moreover, the combination of BLI with phasor analysis has demonstrated the possibility to distinguish spectrally similar bioluminescent reporters that will bolster future efforts to visualize multiple molecular events in real-time (Yao et al., 2022).

4. Conclusions

The increased accuracy of microfluidic organ-on-chip technologies on predicting in vivo outcomes urgently demands the incorporation of biosensing features that allow continuous, non-invasive, and in-situ imaging of cells, tissues, or miniaturized organs. Optical biosensors offer new unexplored pathways for integration in on-chip platforms, enabling imaging at single cell resolution. Altogether, in this work, we successfully integrated BLI for the first time in an on-chip platform, featuring single-cell level resolution and high signal-to-noise ratio (SNR) as

**Fig. 5.** BLI imaging offers superior signal-to-noise ratios for on-chip platform integration. Representative brightfield, Fluorescence, and Bioluminescent microscopy images, and respective SNR calculations expressed in relative light units (RLU) of HEK-NanoLuc cells encapsulated in (a-a’) Agarose, (b-b’) GelMA and (c-c’) Fibrin hydrogels and integrated on the on-chip platform. Single cell resolution was achieved using a 10x lens magnification. Scale bar is 50 μm. Statistical analysis of data was performed using one-way ANOVA followed by t-test (**p < 0.0033; ***p < 0.0008; ****p < 0.00008). Error bars represents ± SD.
incorporation of Nanoluc-bioengineered reporter cells on-chip can

Data availability

Data availability: The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2023.115510.

References

Nuno Araújo-Gomes: Investigation, Writing - Original Draft, Formal Analysis; Gisella Zamboni: Writing - Original Draft, Formal Analysis

CRediT authorship contribution statement


Appendix A. Supplementary data

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