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Highly sensitive luciferase-based assay with red fluorescent protein expression for accurate quantitative monitoring and real-time visualization of cell invasion

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ABSTRACT

Background: Traditional migration and invasion assays like scratch, Transwell, and Boyden chamber are widely used but have disadvantages such as being time-consuming, lacking real-time monitoring, and relying on endpoint measurements. We addressed these limitations by developing a novel fluorescent and luciferase-based invasion assay.

Materials and methods: Three stable cell lines co-expressing the red fluorescent protein dTomato, and secreting luciferase were generated based on Caco-2, MDA-MB-231 and HEK293T cells. Transwell chamber membranes were coated with Matrigel for invasion assay, onto which the modified cells were seeded. To simulate non-invasive and invasive conditions, chambers were incubated for 48 h in FBS-free or FBS-supplemented medium. Following incubation, the Matrigel along with non-invasive cells were removed, and the chambers washed before being transferred into fresh media for 24 h allowing the cells to secrete luciferase. Luciferase activity was measured and compared to traditional cell counting invasion assay, with further confirmations through Z-stacking and microscopic fluorescent imaging.

Results: Our results demonstrated that luciferase activity accurately correlates with cell count. Applying luciferase efficiently quantifies variation in cell invasion with higher sensitivity, hence improving detection of low-level invasion as compared to cell counting techniques based on nuclear staining. The expression of the fluorescent dTomato protein proved ideal for real-time visualization of invading cells.

Conclusion: Overall, using luciferase and dTomato co-expressing cells for invasion assay showed reliable and accurate measurements of variations in cell invasion patterns. Introducing these cells reduced time-consuming steps, improved sensitivity, and endpoints measurements, while being capable of real-time visualization, providing advantages over traditional methods.

1. Introduction

Cell migration and invasion are essential mechanisms involved in multiple biological processes such as embryonic development, tissue repair, immune responses, and progression of pathological diseases [1]. These processes involve cellular movements enabling the ability to reach distant tissues, which occur in both two-dimensional (**2D**) and three-dimensional (**3D**) environments [2]. In a 2D plane, cells adhere and move across flat tissue areas, whereas in a 3D plane, cells interact and penetrate extracellular matrix barriers [3]. Therefore, these mechanisms emphasize the essential role of cellular movement, relying on the cells capacity to adapt and reposition precisely within various settings to carry out specific functions effectively. [4]. Tools for investigating the migration and invasion of 2D and 3D *in vitro* tissue models have been implemented and designed to replicate identical characteristics observed in *in vivo* environments [2]. The assessment and quantification of these cellular models play a significant role in advancing our understanding of cellular behavior in the microenvironment and in the discovery of novel therapeutic agents and treatment possibilities [5].

Well-established methods such as scratch assays, Transwell

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migration, and Boyden Chamber assays, are frequently utilized tools for studying invasion and migration [6]. These techniques may be used for the rapid evaluation of therapeutic agents, innovative approaches in pathophysiological diagnosis, and screening of new molecules that induce malignant migration and invasion [7]. However, these methods are not without disadvantages. Scratch assays have inconsistencies due to irregular wound borders such as variations in scratch width [8]. Transwell and Boyden Chamber assays rely on porous filter membranes and extracellular matrix proteins to establish a physical barrier, facilitating the assessment of migratory behavior and invasiveness. In these assays, the traditional examination of cells encompasses fixation and staining followed by manual cell counting procedures, which involve time-consuming processes that are prone to inaccuracies [6]. Reliance on manual counting impacts overall reliability and therefore, additional tools and cell-counting software are often utilized [9]. Despite advancements, these techniques still lack real-time monitoring and endpoint measurements, which would be beneficial for efficiently capturing and measuring migration and invasion [10].

Among the current methods for studying cellular behavior, measuring luciferase activity has been established as a promising approach for quantifying cell numbers and assessing cellular responses [11,12]. Although the use of luciferase as a reporter gene in cellular studies is well-recognized, the conventional techniques are a multi-step process requiring cell lysate harvesting to obtain luciferase [13]. However, in 2022 Jessen et al. [14], established CRISPR-modified Lucia-luciferase reporter gene cells that secrete luciferase into the growth media, rather than accumulating it intracellularly. This method showed a direct correlation of seeded cell number against luciferase signal. Facilitating a high-throughput approach for measuring luciferase activity and allowed the examination of several media samples during adhesion and proliferation, while preserving a high sensitivity. This proved a reliable tool for the quantitative evaluation of cell count and cellular responses, which is capable of monitoring behavior in real-time, maintaining an easy-to-perform assay and, eliminated the need for cell counting, and obtaining luciferase from cell lysis [14].

Despite the fact that this method excels in capturing adhesion and proliferation, no similar approach applies luciferase to measure migration and invasion. Therefore, in this study, we aimed to design a luciferase-based assay for measuring cell invasion with the co-expression of red fluorescent protein (RFP) dTomato, introducing real-time visualization, hence, expanding on the principles established by Jessen et al. [14]. Developing an assay that enhances accuracy and reproducibility, while also improving real-time monitoring and endpoint measurements, thus overcoming previous limitations.

2. Material and methods

2.1. Cell culture, construction of reporter plasmid and transfection

To generate a plasmid capable of co-expressing secreted luciferase and RFP, and integrating into the safe harbor AAVS1 locus via CRISPR/Cas9 editing, a fragment containing the IRES and the dTomato gene from the pHIV-dTomato plasmid (a gift from Bryan Welm; Addgene plasmid #21374) was cloned into the pSelect-Zeo-Lucia-AAVS1 plasmid (Jessen et al., 2022) using the In-Fusion Cloning Kit (Takara Bio Inc.), resulting in the plasmid pML Lucia dTomato. The plasmid map and GenBank sequence file are available as supplemental files (Supplemental_Figure_1 and pML Lucia dTomato). The pML Lucia dTomato plasmid was co-transfected with the AAVS1-targeting CRISPR/Cas9 plasmid (AAVS1 T2 CRISPR in pX330; Addgene plasmid #72833) into the human colon cancer cell line Caco-2,the human breast cancer cell line MDA-MB-231 and the human embryonic kidney cell line HEK293T. Stable polyclonal cell lines secreting Lucia-luciferase and expressing dTomato were generated under Zeocin selection, as described by Jessen et al. (2022).

2.2. Cell line cultivation and serum-starvation

All three cell lines were maintained in T75 flasks containing Dulbecco's Modified Eagle's Medium (DMEM) composed of 0.862 g/L Glutamax-1, 4.5 g/L D-Glucose, and pyruvate (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) growth medium. The medium was supplemented with 10 % Fetal Bovine Serum (FBS) (Hyclone™, Cytiva, Marlborough, MA, USA) and 100 units/mL penicillin and streptomycin (Gibco™). Cells were incubated at 37 °C in 5 % CO_2 and passaged twice a week every 3–4 days. For experimental purposes, cells were grown to 70–80 % confluence, then serum-starved by aspirating the medium, and washed thoroughly with phosphate buffered saline (PBS) (Gibco™) to remove any residues of FBS. Then, DMEM composed of 0.862 g/L Glutamax-1, 4.5 g/L D-Glucose, and 100 units/mL penicillin and streptomycin without pyruvate and FBS was reintroduced. Cells were serum-starved for a period of 6- to 8 h.

2.3. Matrigel coating of Transwell chambers

Invasion assay was performed using 24-well Transwell chambers with a pore size of 8.0 μm (9328002 CellQUART®, Northeim, Germany). The surface membranes of the chambers were coated with Cultrex® RGF Basement Membrane Extract, Type 2, Select Matrigel (R&D Systems, Minneapolis, MN, USA), a commonly used Matrigel for cell culture applications, including cell invasion studies. To ensure optimal coating, Matrigel was thawed in an ice bath to prevent early solidification at room temperature. Once thawed, Matrigel was diluted in cold FBS-free media to establish a final concentration compatible with the cell lines. This was achieved by diluting a Matrigel stock solution ranging from 8 to 12 mg/mL to final concentrations of 4–6 mg/mL (1:2 ratio) for MDA-MB-231 cells and 2–4 mg/mL (1:3 ratio) for Caco-2 and HEK293T cells. The diluted Matrigel/media mix was resuspended to generate a uniform solution. To avoid damaging the Transwell membrane and the formation of air bubbles while maintaining the integrity of the Matrigel, an even level of coating applied with 70 μL per chamber was carefully pipetted onto the centre of the membrane using pre-chilled tips that had been stored in the freezer. All coated Transwell chambers were incubated under cell culture conditions for the same period as the serum-starvation, allowing the Matrigel to solidify before use.

2.4. Invasion assay

After serum-starvation, the cells were prepared for seeding in DMEM composed of 0.862 g/L Glutamax-1, 4.5 g/L D-glucose, and 100 units/mL penicillin and streptomycin without pyruvate or FBS. The cells were seeded according to cell line onto the Matrigel layer in a total volume of 200 μL FBS-free medium. All Transwell chambers were immersed in 1 mL containing either FBS-free media (control) to simulate a non-invasive environment or media supplemented with 10 % FBS (invasion) to promote cell migration and invasion, and each experiment was performed in four replicates. The chambers were incubated for 48 h under standard cell culture conditions, then media was aspirated, and non-invasive cells along with Matrigel were removed using one end of a sterile cotton swab. All chambers were then washed twice in PBS and swabbed with the other end, followed by an additional washing twice in PBS to remove any Matrigel and non-invasive cell debris, while the cells adhering to the outside of the Transwell chamber remained undisturbed. Washed Transwell chambers were transferred to fresh media containing 500 μL of 10 % FBS and incubated for a further 24 h to allow the remaining cells to secrete luciferase into the surrounding medium. Finally, the enzymatic activity was quantified using media samples.

2.5. Measurement of luciferase

Luciferase activity was measured by transferring 20 μL media samples to a 96-well microplate. A stock solution of QUANTI-Luc

(InvivoGen) assay reagent was made according to the manufacturer's protocol, which was further diluted in 5x distilled water and allowed to reach room temperature to ensure optimal enzymatic activity. All measurements were obtained on a GloMax Navigator microplate luminometer (Promega, Madison, WI, USA) by injecting 50 μL of QUANTI-Luc per well at a rate of 250 μL/s and taking endpoint measurement with a 4 s start time and a 0.1 s reading frame. Luciferase activity was recorded as relative light units (RLU) values.

2.6. Visualization and counting

For cells expression RFP dTomato, real time imaging of cell invasion was monitored on an EVOS M5000 microscope imaging system (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) equipped with an LED light cube, RFP 2.0 filter (Invitrogen) with excitation and emission (wavelength/bandwidth) of 542/20 and 593/40 nm, respectively. Z-stacking imaging was performed by capturing 100 images across the Z-axis after 0, 24 and 48 h, which were later processed using the microscopy imaging software Imaris 10. Then to validate the cell count of adherent cells on the outside of the Transwell chamber, hereafter also referred to as invasive cells, the following was undertaken. After completion of the luciferase measurements, the invasive cells were fixed in 10 % formaldehyde for 2 min and then permeabilized using 100 % methanol for 20 min. The cells were then stained with 2 μg/mL DAPI for 15 min to visualize nuclei. Between each step, the Transwell chamber was washed twice with PBS. For microscopy and image analysis, the Transwell chambers were mounted onto a glass slide. Images were acquired on an EVOS equipped with an LED light cube, DAPI 2.0 filter (Invitrogen), with excitation and emission (wavelength/bandwidth) at 357/44 and 447/60 nm. A 10× magnification with 4x/10 phase contrast was used to capture both Z-stacking and field of view (FOV) images of the Transwell chambers.

2.7. Statistical analysis

CellProfiler image analysis software was used to segment nuclei [15], and the mean number of invasive cells was quantified for each Transwell chamber based on 5–6 FOV randomly selected image samples. Statistical analysis for luciferase-based invasion assay was performed in GraphPad 10, wherein the level of significance was set at a p-value of 0.05. Differences are marked as p-value 0.05* and p-value 0.0001**** in the graphical representation. The statistical difference in luciferase activity between control and invasion was determined using an unpaired *t*-test for both Caco-2 and MDA-MB-231 cells. Linear regression analysis evaluated the relationship between cell count and luciferase activity where the best-fit line was calculated using the least squares method, and the goodness of fit was assessed by coefficient of determination (R^2).

3. Results and discussion

In this study, we aimed to develop a luciferase-based assay that captured *in vitro* cell invasion and was to be performed according to Fig. 1. The initial goals were to enhance sensitivity, introduce real-time monitoring, and reduce the time-consuming steps associated with conventional cell counting techniques. To achieve this, cells capable of co-expressing RFP dTomato and luciferase to generate visualization and continuous secretion of luciferase into the surrounding medium were applied. However, as the luciferase diffuses and accumulates in both the upper and lower medium chambers, it was essential to remove all the upper non-invasive cells, wash the chambers with PBS, and transfer the Transwell chambers into fresh media when designing the assay. This process ensured that luciferase activity measurements corresponded exclusively to a population of invasive cells, making it possible to collect media samples containing luciferase, and thereby quantify the number of cells present on the outside of the Transwell membrane.

3.1. Evaluating luciferase as a measurement for cell invasion

In order to demonstrate the usability of the luciferase-based assay for measuring cell invasion, we explored the relationship between luciferase activity and cell count to evaluate whether luciferase measurements could serve as an alternative for cell counting. Therefore, we assessed the correlation between cell counts, determined using nuclear staining and luciferase activity, by screening a wide range of seeding densities between 10,000 and 60,000 MDA-MB-231 cells, collecting luciferase measurements, and visualizing the nuclei of adherent cells on

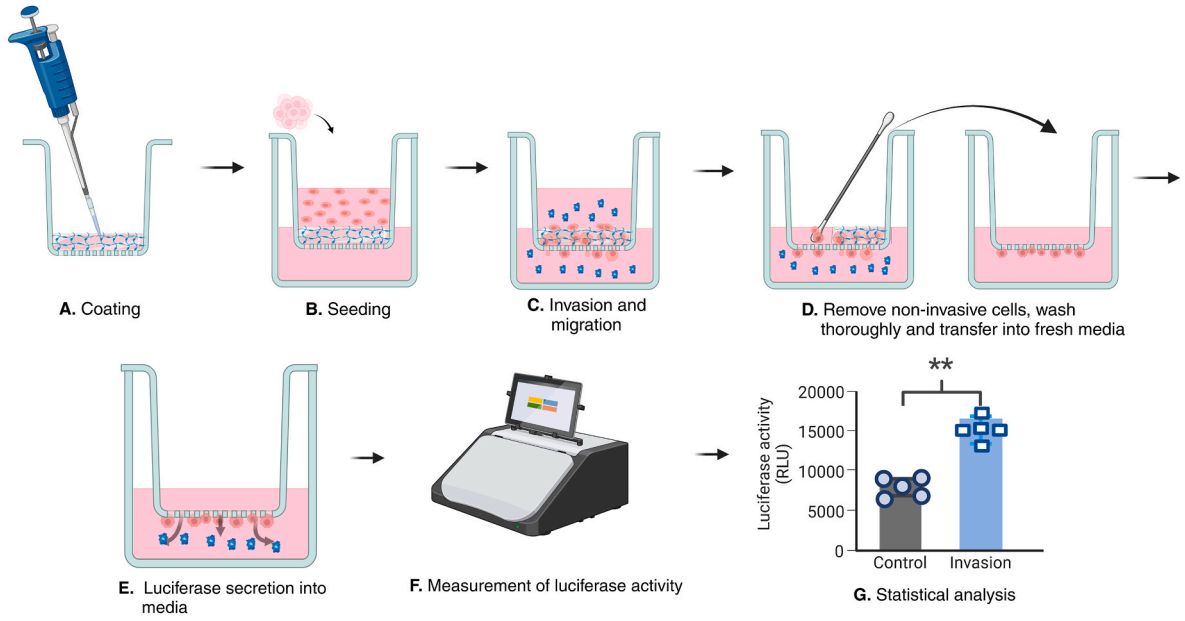

Fig. 1. Schematic illustration of luciferase-based invasion assay: **A.** Coating the Transwell chambers membranes with Matrigel. **B.** Seeding of the luciferase secreting cells. **C.** Incubation period to allow invasion and migration, penetrating Matrigel and crossing of Transwell membrane pores; **D.** Removing matrigel and non-invasive cells with cotton swab followed by washing in PBS. Transfer into fresh media; **E.** Luciferase is secreted into the media; **F.** Measurement of luciferase activity. **G.** Comparing differences between control and invasion.

Transwell membranes using DAPI staining, as shown in Fig. 2.

All captured FOV images were processed using CellProfiler to identify nuclei and quantify the cell count for each individual Transwell chamber. Two representative FOV images were selected from the control and invasion groups to visualize the differences between them, each group seeded with 20,000 cells. In (Fig. 2A), observing a lack of cell invasion, whereas for (Fig. 2B) showing an abundance of cell that crossed the Transwell membrane. Based upon all our captured images, we observed a clear difference in cell distribution between the control and invasion, successfully demonstrating variation in invasiveness between the two.

To establish a correlation curve where an increasing cell count that corresponds proportionally with luciferase activity, the relationship between 18 different samples was compared (Fig. 3). The results revealed a strong positive correlation between RLU signals and cell count, in which an R^2-value of 0.986 across all seeding densities was achieved. This confirmed that quantification of luciferase activity was well-suited as a substitution for traditional staining based cell counting when doing invasion assays. The novel method can be used alone to efficiently measure the cell invasion or as a complementary tool to support more accurate quantification of cellular behaviors and mechanisms such as invasion and migration.

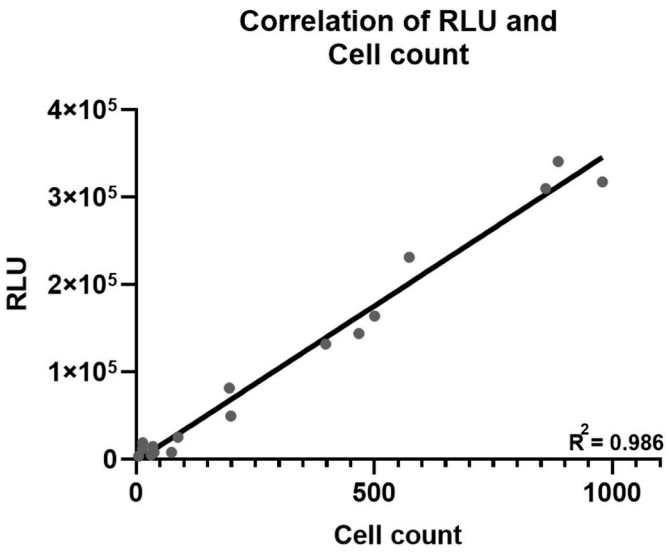

Fig. 3. Correlation of luciferase activity (RLU) and cell counts: Scatter plot of cell count (x-axis) and RLU (y-axis) made after conducting several invasion assays and collecting media samples followed by cell counting. Seeding densities ranged between 10,000, 20,000, 30,000, 40,000, 50,000, and 60,000 cells.

3.2. Luciferase-based invasion assay

To highlight that the application of luciferase-secreting cells is a valid and reliable approach for measuring *in vitro* cell invasion, we compared luciferase activity in a control lacking FBS against that in media containing 10 % FBS, expecting to promote cell motility as a response. Additionally, we performed the assay including different cell lines using 40,000 Caco-2 and 20,000 MDA-MB-231 cells. Caco-2 cells represent less aggressive cancer cells, exhibiting lower invasive capabilities, whereas MDA-MB-231 is known to be highly invasive [16–18]. As shown in Fig. 4, both cell lines showed consistently lower luciferase activity, indicating minimal cellular invasion without the presence of FBS. In the presence of 10 % FBS the Caco-2 cells (Fig. 4A) had a significant difference in invasion as compared to that in the control, with a p-value of 0.0001. The MDA-MB-231 cells (Fig. 4B), which are known for being more prone to invade, produced a 10-fold higher signal compared to Caco-2, but still displayed a significant increase in invasion with a p-value of 0.0286.

These results highlighted MDA-MB-231 invasive properties, since a greater luciferase signal corresponds with more cells being present on the Transwell membrane. Caco-2 produced low biological variance, resulting in consistent reproducible signals between all replicates of the two groups. The MDA-MB-231 cells, on the other hand had greater invasion potential, hence also susceptible to higher variability observed in the relatively larger deviations in luciferase measurements.

The fluctuations in luciferase signal across the cell lines, especially the variability seen for MDA-MB-231 as compared to the more consistent signals for Caco-2, suggest a dynamic luciferase expression profile specific for each individual cell line. In comparison to Caco-2 the MDA-MB-231 cells exhibit enhanced invasive properties, and thus higher luciferase signals may be caused by an elevated promoter activity in MDA-MB-231, potentially influenced from transcription factors. As a result, even minimal variations in successfully migrated cells across the replicates would yield deviated signals, emphasizing the sensitivity of the method. Therefore, the Caco-2 cells, which expressed a more consistent level of luciferase between replicates, resulted in a stronger statistical significance amongst the two cell lines.

However, despite this variability, our assay applying luciferase provided signals across all replicates that still yielded a significant level of difference between the measurements for both cell lines. In contrast to our findings from quantifying invasion by counting cells, we were unable to localize cells in the control group using DAPI.

Hence, the use of luciferase-secreting cells provided a more sensitive

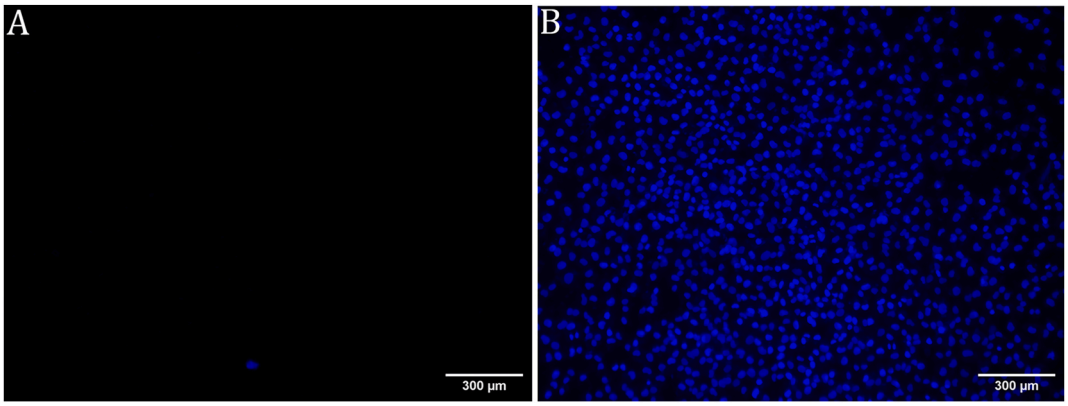

Fig. 2. Images of MDA-MB-231 using DAPI staining: Invading cells were assessed using 2 µg/mL DAPI solution to visualize nuclei. Following staining procedure, Transwell membranes were mounted on a glass slide and 5–6 FOV were captured with 10× magnification. Scale bar = 300 µm; **A.** Limited invasion observed for control with 0 % FBS; **B**. Invasion observed with 10 % FBS showing abundant stained nuclei.

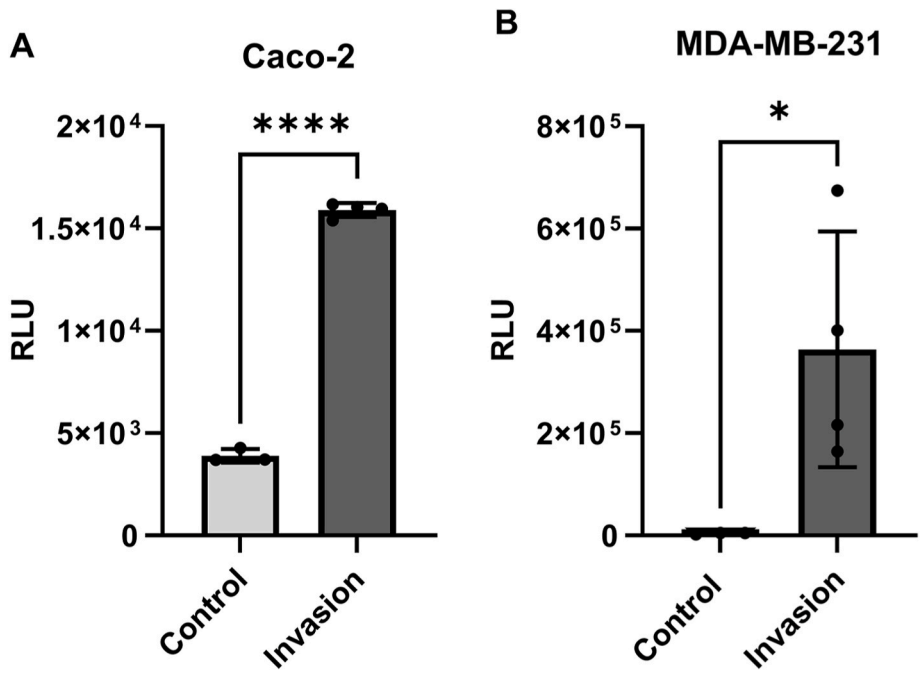

Fig. 4. Luciferase-based invasion assay: Comparison of cell invasion between control (0 % FBS) and invasion (10 % FBS) for luciferase secreting Caco-2 and MDA-MB-231 cell lines (n = 4). Luciferase activity was measured in RLU, and invasiveness was determined. (*) p-value<0.05, (****) p-value<0.0001. Intra-assay coefficient of variation (CV%) was for Caco-2 control = 7.3 % and invasion = 1.87 % and for MDA-MB-231 control = 26.9 % and invasion 64.7 %.

approach for assessing *in vitro* cell invasion compared to nuclear staining. The applicability of luciferase maintained a reproducible signal for both control and invasion confirmed by intra-assay coefficient variation of control = 7.3 % and invasion 1.87 % for Caco-2. Although, for MDA-MB-231 cells a higher CV% for control (26.9 %) and invasion (64 %) indicated a reduced reliability for this cell line. Overall, these findings suggest that luciferase-based measurements may be more suitable for cell lines expression lower invasive properties such as the Caco-2.

3.3. Real-time visualization of cell invasion

To evaluate the capabilities of RFP dTomato for real-time visualization of cell invasion, Z-stacking images were captured for both control and invasion conditions using 20,000 MDA-MB-231 cells and 40,000 HEK293T cells. Each stack composed of 100 images, covering a depth ranging from 4037 μm (top) to 2878 μm (bottom), with a step size of 8–10 μm between each individual image. To monitor the invasion, stacks were captured at 0, 24 and 48 h. Identical settings were applied across both groups to ensure consistency when visualized. Compelling stacks into 3D reconstructions were generated in the software Imaris, as illustrated in Fig. 5 and Fig. 6.

Fluorescence intensity observed along the Z-axis indicated the progression of invasion. Amongst all images the most notable difference was observed for the MDA-MB-231 cells (Fig. 5). In control condition (Fig. 5A) cells remained localized at the Matrigel surface. In contrast, serum-induced cells (Fig. 5B) initially adhered to the surface, but after 24 h and 48 h clearly deviating from the uniform distribution seen in the control group. This demonstrated that serum stimulation induced an invasive response increasing the activity as intended.

In comparison, the HEK293T cells showed no apparent invasion under either condition (Fig. 6A–B). Even when induced by serum, these cells remained at the top of the Matrigel, which can be linked to their limited invasive capacity to degrade the matrix proteins [19]. Moreover, HEK293T cells tended to attach to one another forming larger clusters, especially notable for the once that had been serum induced. These clusters generated fluorescent interference that hindered a clear observation in the Z-axis.

In addition, we captured images after removing non-invasive cells, and while the HEK293T cells did not pass through the Transwell membrane (Fig. 6C–D). The MDA-MB-231 cells (Fig. 5C–D) exhibited low fluorescence intensity in control compared to a much greater intensity in the invasion reflected by the number of cells present on the outside of the Transwell chamber, which aligned with our Z-stack images. This matched the pattern we previously observed in our DAPI examinations. Hence, the application of RFP dTomato expressing cells effectively complements our luciferase-based assay, facilitating real-time imaging of cell invasion progression through Z-stacking, while providing visualization of invasive cells that passed the Transwell membrane, which is more easily acquired than the DAPI technique.

3.4. Future applications

Three cell lines representing colon, breast and kidney, were included in this study, providing a basis for cellular examination. Ideally, the developed assay could be applied for screening anti-metastatic drugs. However, expanding the repertoire of cell lines expression RFP dTomato and luciferase would be beneficial for broadening the scope of its applications. For instance, future studies could incorporate these cells into 3D models, such as patient derived organoids, which encompass a tissue-like environment, hence create systems with clinical relevance. In addition, while Transwell chambers served as a useful starting point, the simplicity of luciferase measurements could be integrated into dynamic platforms such as microfluidic and organ-on-a-chip, potentially making quantitative readouts in these complex models easier and more practical. Alternatively, beyond the scope of cellular invasion and migration, this advancement could be adapted for monitoring immune cells migration, screening antimicrobial agents in co-culture models and wound healing assay assessing regeneration in 2D environments. Covering a broad range of biomedical applications for rapid evaluation of novel strategies, therapeutic agents and pathological diagnostics.

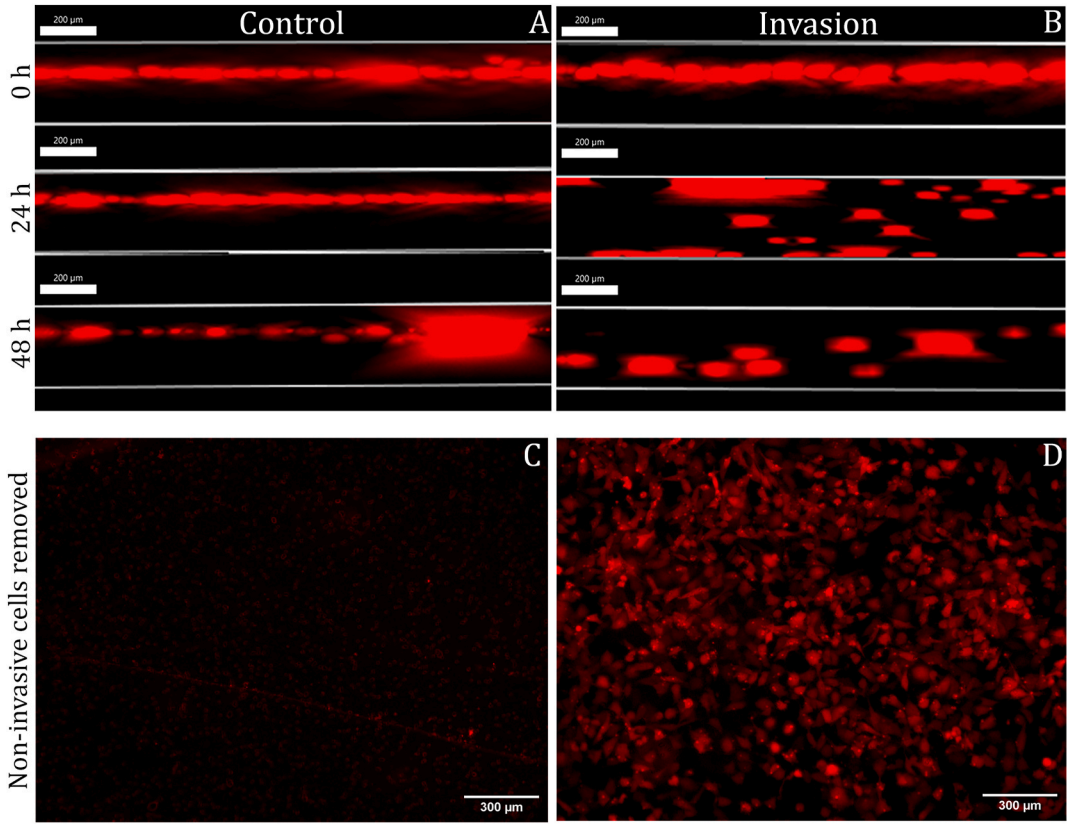

Fig. 5. Real-time images captured of MDA-MB-231 with RFP dTomato expression: Z-stacking images **A** and **B** were captured at 0, 24 and 48 h, covering 100 images in the Z-plane from top to bottom of the Matrigel layer, which were compiled into 3D using Imaris software. Scale bar = 200 µm. Then non-invasive cells were removed, and images **C** and **D** were captured for the XY-plane to locate remaining invasive cells adhering outside the Transwell membrane. Scale bar = 300 µm. All images were captured using 10× magnification. **A.** Z-stacking for control, cells located in the same plane at 0, 24 and 48 h indicating low invasive motility. **B.** Z-stacking for invading cells passing into the Matrigel, as evidence by the scattering observed after 24 and 48 h, indicating high activity. **C.** Control showing an absence of cells that had crossed the Transwell membrane. **D.** Invasion with high number of cells that had crossed the Transwell membrane.

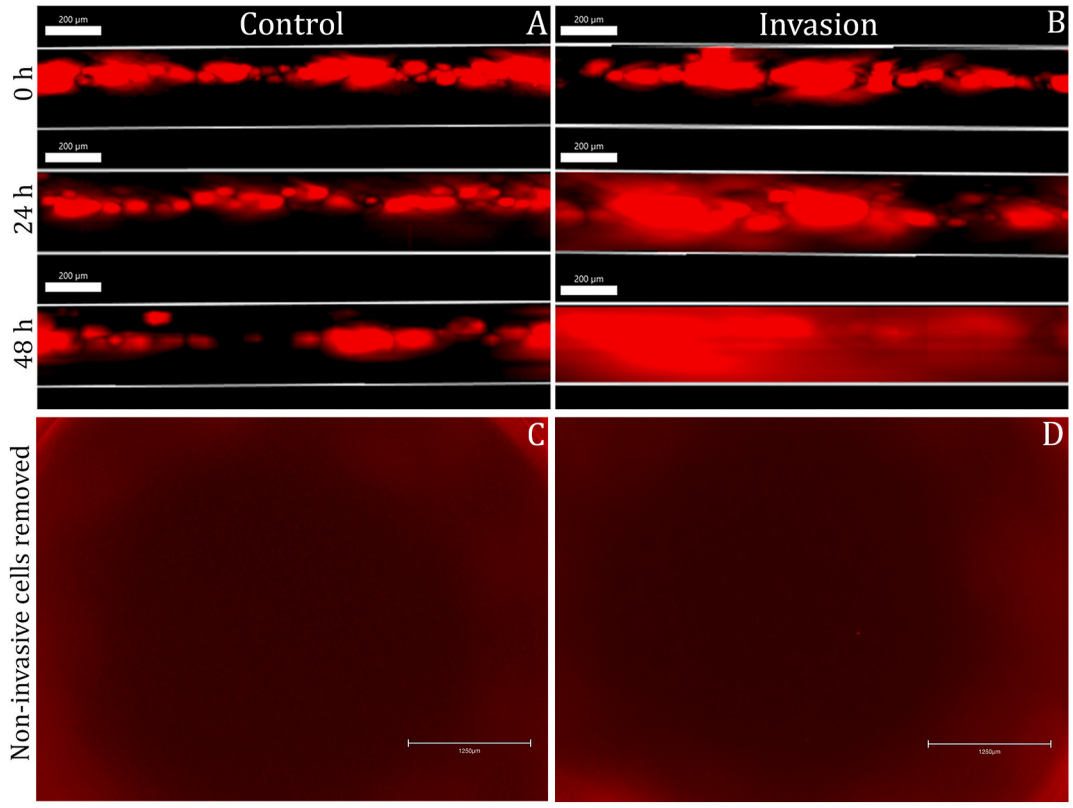

Fig. 6. Real-time images captured of HEK293T with RFP dTomato expression: Z-stacking images **A** and **B** were captured at 0, 24, and 48 h, covering 100 images per stack in the Z-plane from top to bottom of the Matrigel layer, and subsequently compiled into 3D using Imaris software. Scale bar = 200 µm. After completion the non-invasive cells were removed, and images **C** and **D** were captured to locate remaining cells outside the Transwell membrane in the XY-plane. Scale bar = 1250 µm. Z-stacking images were captured using 10× magnification, while 2× magnification were used for locating any remaining invasive cells. **A.** Z-stacking for control, cells located on top of the Matrigel throughout the 0, 24 and 48 h. **B.** Z-Stacking for invasion, showing low-invasive activity, remained positioned at the top of the Matrigel throughout the 48 h. However, gradually formed larger clusters as reflected by an increased RFP dTomato intensity after 48 h. **C.** Control showing an absence of cells that had crossed the Transwell membrane. **D.** Low-invasion confirmed by the absence of cells that had crossed the Transwell membrane, even when induced with serum.

4. Conclusion

This study has demonstrated that the RFP dTomato combined with a luciferase-based invasion assay is a reliable method for accurate quantification of cell invasion. This assay overcomes several limitations of traditional cell count-based methods by simplifying experimental procedures, reducing the time compared to the conventional cell counting techniques, and enhancing the accuracy of endpoint measurements. The developed luciferase-based assay also easily accommodates more samples for analysis, enhancing sensitivity, and in combination with RFP dTomato makes it possible to real-time monitor invasion as images and samples can be collected at any given interval during the assay. Overall, the application of RFP dTomato- and luciferase-expressing cells can be regarded as a valuable addition to invasion assay for cellular studies.

CRediT authorship contribution statement

Michael Lyngbæk Christensen: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tove Kirkegaard:** Writing – review & editing, Validation, Supervision. **Ismail Gögenur:** Writing – review & editing, Validation, Supervision. **Frederik Diness:** Writing – review & editing, Validation, Supervision, Resources. **Jesper Thorvald Troelsen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Stine Bull Jessen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2025.115986.

Data availability

Data will be made available on request.

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