

Peer Review File

Single-Cell Laser Emitting Cytometry for Label-Free Nucleolus Fingerprinting



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Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

The article described a single cell lasing cytometry for cell nucleolus profiling. The fundamental mechanism is the "focusing effect" by nucleoli due to their higher refractive index effectively creating a lens within nucleus. By sandwiching the cells within a Fabry-Perot resonator with a fluorescent thin layer as gain media, the "focusing effect" reduced the lasing threshold to allow significant emission from the Fabry-Perot resonator compared with non-nucleoli region. The fundamental mechanism is considered sound, with numerical simulation and extensive demonstrations in spreading cells, as well as suspended cells. The article also demonstrated flow cytometry that quantifies the emission spectrum, which was further used for cell type classification based on profiling. While the technical content is strong and the study is considered rigorously performed, there are concerns whether the technique would make significant impact in the form of flow cytometry, for the following reasons/comments.

1. What is the throughput for the proposed cell cytometry? The camera exposure was 10ms exposure, and that is consider very slow in comparison to conventional flow cytometry.
2. The photobleaching for the fluorescence polystyrene is a significant limitation for the practical use of the device (<5 mins total exposure to avoid photobleaching). Unless there is a way to recharge the fluorescence layer, it is hard to envision a prolong use for the device.
3. The multiple laser peaks from larger nucleoli is reasoned to be HRIUs (high refractive index units). It seems more like lasing with multimode?
4. The approach is specifically developed to profiling nucleoli. While the case is made in the label-free approach without introducing fluorescent target, the advantage is offset by the exclusive design only for nucleoli. I found it less compelling with more conventional imaging based cytometry, using live cell nucleoli dyes (e.g. <https://www.enzolifesciences.com/ENZ-51009/nucleolar-id-green-detection-kit/>).

Reviewer #2:

Remarks to the Author:

This paper reports the establishment of a method for the analysis of nucleoli using laser emission in a Fabry-Perot photonic resonator. This method enables nucleolus-selective lasing by taking advantage of the lower laser emission threshold derived from the higher refractive index of the nucleolus. The authors showed that this method of analyzing laser emission images and spectral fingerprints allows for a more accurate assessment of nucleolus number, morphology, fluid-like dynamics, and heterogeneity than the conventional methods. The development of detection methods for nucleolus state changes is important because they are used in the diagnosis of cancer pathology and as indicators of cellular stress. The advantage of being able to assess the quality and quantity of nucleoli in individual living cells without the need for specialized stains or the introduction of exogenous fluorescent proteins makes this technique an effective tool for drug evaluation and the study of diseases such as cancer and neurodegenerative disorders. In particular, analysis by flow cytometry has great potential. However, this method is not suitable for the detection of cells in tissues because it can only be applied to individually isolated cells such as cultured cells, making it difficult to use in histopathology, which is the most important and necessary application method. In this regard, the evaluation of this report must be lowered. Therefore, this paper is not recommended for publication in the Journal of Nature Communications.

Specific comments:

1. Figure 4-f-II: What is the laser emission observed in the metaphase detecting? Is it related to the nucleolus or is it laser emission resulting from an increase in refractive index due to chromosome aggregation?

2. Figure 6: Data on the nucleolus of various cultured cells should be examined for their validity by comparing them with observations made by other methods.

Reviewer #3:

Remarks to the Author:

The authors describe optical measurements of living cells embedded in a DBR laser cavity. The laser oscillation is facilitated particularly at the nucleolus, where the nucleolus acts as high refractive index lens, enabling label-free imaging with high spatial resolution. The applicability of this method to the suspended cells and flow cytometry is also demonstrated. The achievements are interesting, and the novelty is clear. However, there are several points to be clarified:

1. Experimental procedures for laser spectroscopic characterization and flow cytometry.

The experimental procedures lack some essential information. Particularly, information about the laser scanning should be added. The procedure of the flow cytometry is also unclear. How wide is the channel? How did the authors scan the excitation laser while flowing the cells? How did the authors ensure that all flowing cells were irradiated with the same power? How did the authors irradiate the excitation laser at each area for 5 min while flowing the cells? Add laser images taken outside of the cells and the outside of the nuclei.

2. Discussions about Fig. 4.

Observe the time-course change in the peak wavelength to validate their claim written in page 9 (the laser's high sensitivity to refractive index changes). In addition, repeat the experiments for Fig. 4c and e for several times and give error bars for supporting the quantitative discussion.

3. Cytotoxicity

The description on the cytotoxicity should be written fairly. The laser threshold reported herein is rather high among organic lasers. Thus, the reviewer wonders why such intense laser did not harm the living cells and find that the repetition rate of the excitation laser was so slow (50 Hz) in comparison to the typical pulsed lasers (1 kHz). The authors should make this point clear in the manuscript and fairly evaluate the cytotoxicity of their method in comparison to the other optical techniques.

4. Comparison with the reference 24

Reference 24 (M. C. Gather et al., Nat. Photon. 2011) reports analogous experimental results, where living cell was sandwiched inside a laser cavity. Nonetheless, the laser emission is homogeneous over the whole cell rather than specific to nucleolus. Provide a reasonable explanation for this difference.

5. Novelty of the method

In my opinion, the novelty of the methodology reported in the manuscript is not very high because the method is mostly identical to those written in the reference 24 except the location of the gain. The authors claim that the method is label-free, but it is realized at the expense of the target-specificity. It is in clear contrast to the conventional label-free techniques (Raman microscope, for instance) that have been developed so that they can identify molecules and proteins without label.

6. Errors

The title in the Supplementary Information is different from that of the main text.

Fig. 2e caption, "inserts" --> "Insets"

Line 217, "the laser emission image result" --> "the laser emission image results"

We would like to thank the three reviewers and the editorial office for taking their time and writing constructive comments to improve the quality of our work. Following the advice, we have conducted new experiments and analyses, with the key amendments include:

- 1) Addressing reviewer #2's concern on tissue-level applications: We have tested our technique on both mice colon tumor tissue and healthy tissue. Results indicated that the technique can be well applied in tissues. For the orthogonal validation, we have validated the tissue-application with HE stains, and validated the flow cytometry results with conventional fluorescence imaging.
- 2) Clarifying the novelty concern from Reviewer #3: Reviewer #3 expressed concerns about the similarity to the one described in Reference 24 (Gather, M., et al., Nature Photon., 2011, 5, 406-410). We respectfully disagree and have clarified the significant distinctions and improvements:
 - **Label-free laser emission:** Unlike the referenced study, our technique achieves label-free laser emission, allowing for the analysis of condensate molecules in their native state. Previous studies relied on green fluorescence protein or probe labelling.
 - **Enhanced threshold difference and pure laser emission without high-order modes:** Our design separates the gain medium from the samples, enhancing the threshold difference by 36-fold between the nucleolus and its surroundings. Therefore, it enables the selective lasing emission at nucleolus with primary low-order modes. Thus, the laser emission could reflect the biological information (nucleolus size, etc.). This achieved the link of laser signals with specific cellular structures in label-free manner. The previous study could only achieve homogeneous emissions across the entire cell.
 - **Laser emission flow cytometry:** We achieved the laser emission flow cytometry in a label-free manner, a significant advancement over the static laser emission studied previously.

These innovations have been highlighted and detailed in the revised manuscript.

- 3) Improvements in response to reviewer #1's technical concerns: As for the camera exposure and throughput issues, we have replaced the pulsed laser with a higher repetition rate and upgraded the spectrometer with faster integration speed, achieving a throughput of ~ 2000 eps. As for the photobleaching issue, we have enhanced the effective working time per device to ~10,350 mins by incorporating multiple gain medium layers and multiple pumping positions.
- 4) Addressing concerns on overspecialization: We have emphasized the significance of nucleolus studies, including their role as indicators of cellular stress, models for liquid-liquid phase separation, and as biomarkers for various diseases. As for the advances over conventional imaging approaches, the proposed laser emission cytometry offers label-free analysis, ultrahigh-contrast imaging, and unique lasing spectrum information, which remains challenging for conventional imaging approaches.

We believe our revised manuscript has addressed all the questions and comments from the editor and three referees. Our detailed responses (in black) to the reviewers' comments (in red), and the text change in the revised manuscript (in blue) below.

Summary of major changes:

We added the Supplementary note 1 to highlight the new mechanism in our study and compared our work with previous studies.

In our study, we observed that the lasers selectively emitted from the nucleolar area, with most emissions displaying single-order mode characteristics. We attribute these observations to disparities in laser threshold levels.

The threshold pump power for lasing can be written as Eq. (N1).

$$P_{th} = \frac{h\nu_p}{2\Delta d\sigma_l\tau_f\eta_c[1-\exp(-\alpha\Delta d)]} \cdot \frac{L}{\iiint S(r, \varphi, z)H(r, \varphi, z)dV} \quad (N1)$$

where h is the Plank constant, ν_p is the frequency of the pump light, σ_l is the laser transition cross section, τ_f is the fluorescence lifetime, η_c is the ratio of the pump power coupled in the cavity to the incident pump power, Δd is the length of the gain medium, α is the absorption coefficient for the pump light, L is the round-trip cavity loss, and $S(r, \varphi, z)$ and $H(r, \varphi, z)$ are the normalized three-dimensional intensity distributions of pump light and laser light, respectively, under the cylindrical coordinate system (r, φ, z) . Consider that the length of the gain medium is very small, the following approximation is satisfied:

$$1 - \exp(-\alpha\Delta d) \approx \alpha\Delta d \quad (N2)$$

In consideration of the diminutive length of the cavity, the diffractive effects of the laser light within the cavity can be neglected. Thus, the subsequent approximation can be obtained:

$$\iiint S(r, \varphi, z)H(r, \varphi, z)dV \approx \frac{1}{\Delta d} \iint s(r, \varphi)h(r, \varphi)rdrd\varphi \quad (N3)$$

where $s(r, \varphi)$ and $h(r, \varphi)$ are the normalized two-dimensional intensity distributions of pump light and laser light, respectively, under the polar coordinate system (r, φ) . To further simplify Eq. (N1), we define the effective mode area of a laser mode as Eq. (N4).

$$A = \frac{1}{\iint s(r, \varphi)h(r, \varphi)rdrd\varphi} \quad (N4)$$

Substituting Eqs. (N2) - (N4) into Eq. (N1), we have:

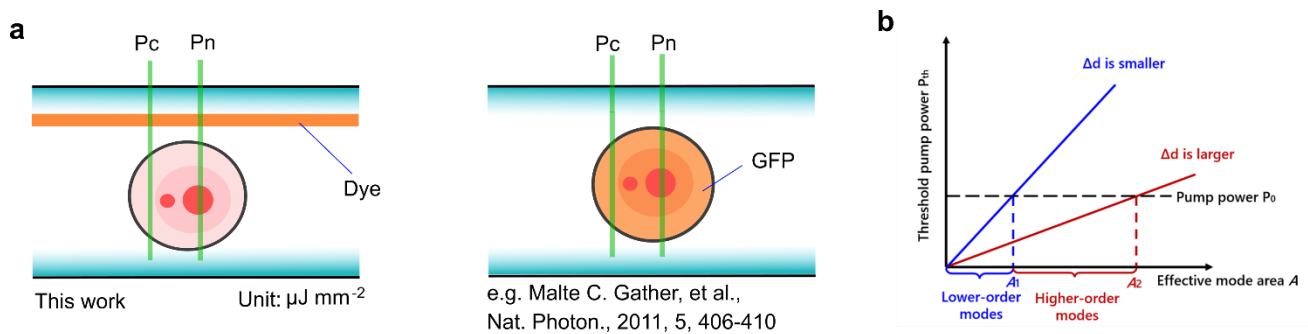
$$P_{th} = \frac{1}{\Delta d} \cdot \frac{h\nu_p LA}{2\alpha\sigma_l\tau_f\eta_c} \quad (N5)$$

Equation (N5) reveals that the threshold pump power is inversely proportional to the length of the gain medium. In the case of our work, the length of the gain medium (PS layer) is substantially reduced. Consequently, the threshold pump power for a specific laser mode is significantly elevated. Additionally, the disparity between the thresholds of lower-order and higher-order laser modes is augmented. The Fig. N1-b elucidates the impact of the gain medium's length (Δd) on the pump threshold. At a given pump power, laser modes with larger effective mode areas can be obtained under a larger Δd . This means that higher-order laser modes can be excited. Conversely, only lower-order laser modes with smaller effective mode areas can be excited under a smaller Δd . Therefore, in

a labeled cell laser, higher-order laser modes can be excited, resulting in an expansive laser pattern that covers the entire cellular structure.

In our work, the laser threshold in the cytoplasm (P_c) reached $\sim 180 \mu\text{J mm}^{-2}$, and the nucleolar area (P_n) exhibited an average threshold of $\sim 40 \mu\text{J mm}^{-2}$. The threshold difference reached $\sim 140 \mu\text{J mm}^{-2}$, facilitating laser emission at the nucleolar area with only lower-order modes. In the case of labeled cells, the threshold in the cytoplasm (P_c) was $\sim 5 \mu\text{J mm}^{-2}$. Assuming a similar lens effect in the nucleolus, the threshold in nucleolar area would be expected to $\sim 1.1 \mu\text{J mm}^{-2}$. The threshold difference was only $3.9 \mu\text{J mm}^{-2}$, resulting in both lower and higher modes over the whole cell.

Therefore, the design of separating gain medium in our work enhances the threshold difference by ~ 36 -fold, markedly improving the discernibility of heterogeneous lasing emissions within cells. Moreover, this configuration ensures predominant laser emissions from the lower-order mode, thereby facilitating spectrum-based analysis.



Supplementary Note Fig. N1. Comparison of our work (label-free cell laser) and the previous work (labeled cell laser) in threshold. (a) Configuration of the devices. In this work, due to the increased threshold, the threshold difference between nucleolar area and cytoplasm significantly increased. In previous research^{1, 2}, the cell was labeled and the gain medium saturated the entire cavity. Therefore, the threshold was relatively low, resulting in a minimal threshold difference. (b) Relationship of threshold, effective mode area, and gain medium distribution.

We added the experiment about tissue-level applications, including the newly added Figure 5 and Supplementary Fig. S15. Fig.1 was also revised to highlight this application. This collaborative work involved Luqi Huang and Prof. I-hsin Su, whose names have been included in the manuscript.

Laser emission from tissues

The nucleolus serves as a pivotal biomarker widely employed in histopathological analyses. we investigated label-free laser emissions at the tissue level. To facilitate comparison, slices of mice colon tumor and healthy tissues ($\sim 10 \mu\text{m}$ thick each) were sandwiched between mirrors. Through adjustment of pump energy, we successfully induced laser emissions from these tissues (Fig. 5a). Contrary to phase contrast imaging, where distinguishing nucleoli can be challenging, laser emissions distinctly highlighted the nucleolar regions. In addition, this method works both fresh

tissues and fixed tissues.

Statistical analysis of the laser emissions from mice tumor and healthy tissues demonstrated a significant difference (Fig. 5b). Notably, tumor tissues exhibited a higher proportion of large lasing spots, indicative of enlarged nucleoli. Furthermore, the density of lasing spots was significantly greater in tumor tissues, with nucleoli in these samples showing decreased circularity, indicative of altered nucleolar morphology associated with pathological conditions. Similar results were observed across multiple sample batches (Fig. S15a-b). To validate the results, we compared them with hematoxylin and eosin (HE) stained images (Fig. S15c-d). Our results exhibited similar nucleolar features achieved by HE stains. These underscore the potential of this technique as an effective tool for tissue-level analysis.

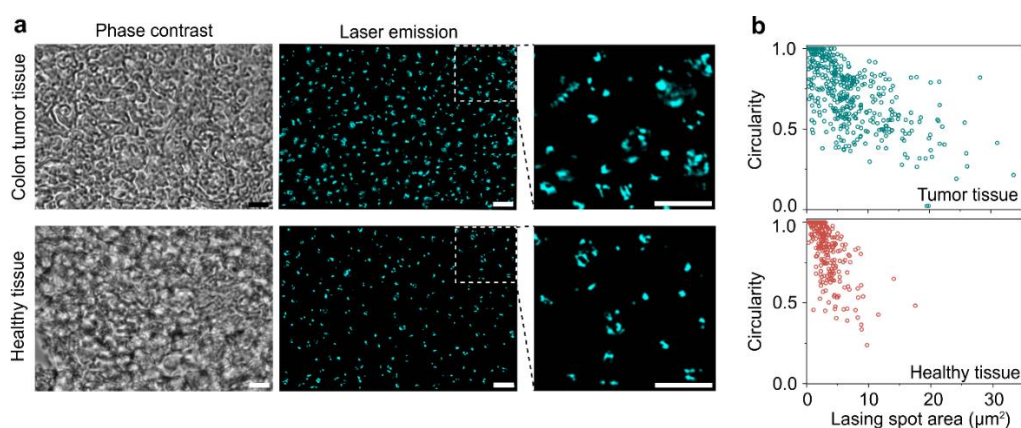
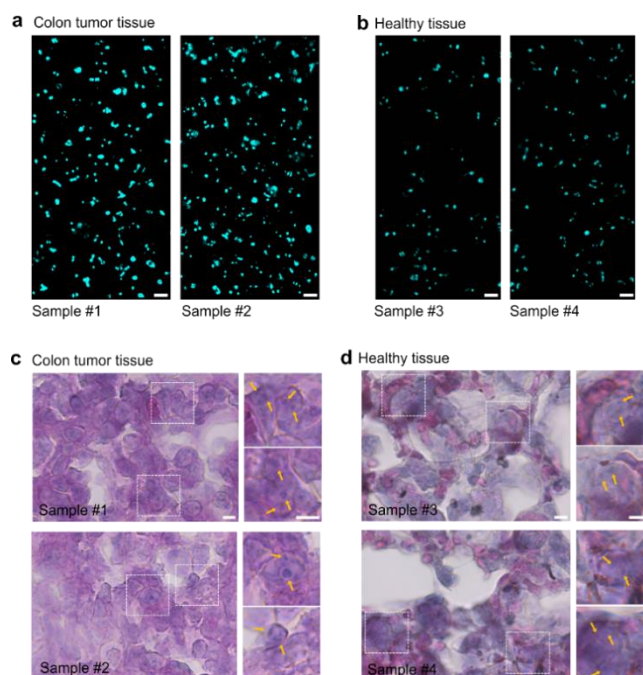


Fig. 5 | Characterization of the laser emission at the tissue level. **a**, Laser emission obtained from mice colon tumor tissue and healthy tissue. Scale bar: 20 μm . **b**, Statistical analysis of the lasing spot area and circularity, correlating with nucleolar morphology variations between tumor and healthy tissues.



Supplementary Fig. S15. Characterization of laser emission from tissues. **(a-b)** Laser emission from the additional tissue samples. Scale bar: 10 μm . **(c-d)** Images of the tissues with HE stains.

Scale bar: 10 μm . Notably, in colon tumor tissue, conspicuous and enlarged nucleoli were observed, some exhibiting altered morphology. Conversely, in healthy tissue, nucleoli were less prominent and displayed smaller size. This indicated that the laser emission technique could revealed similar nucleolar features with simple process and ultrahigh-contrast signals.

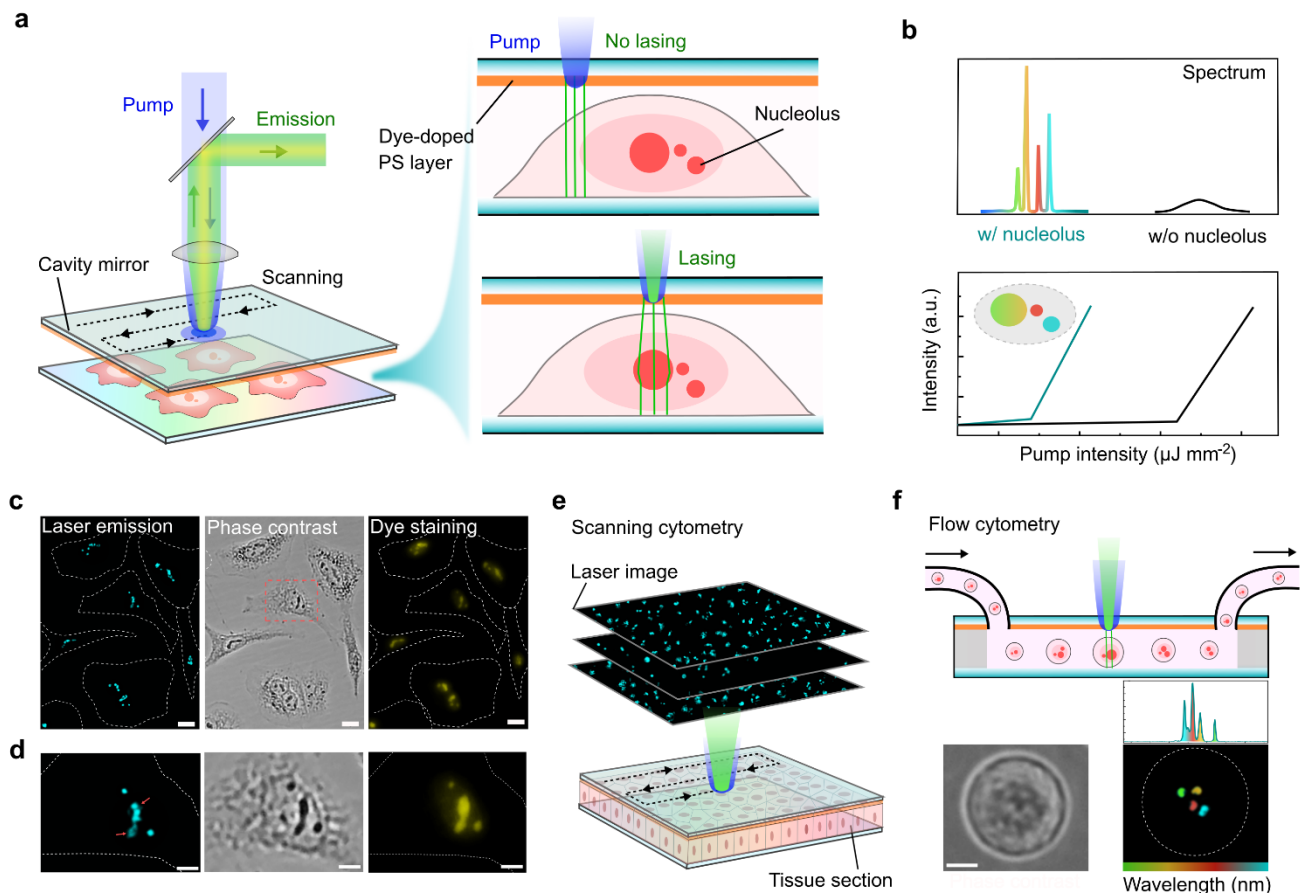
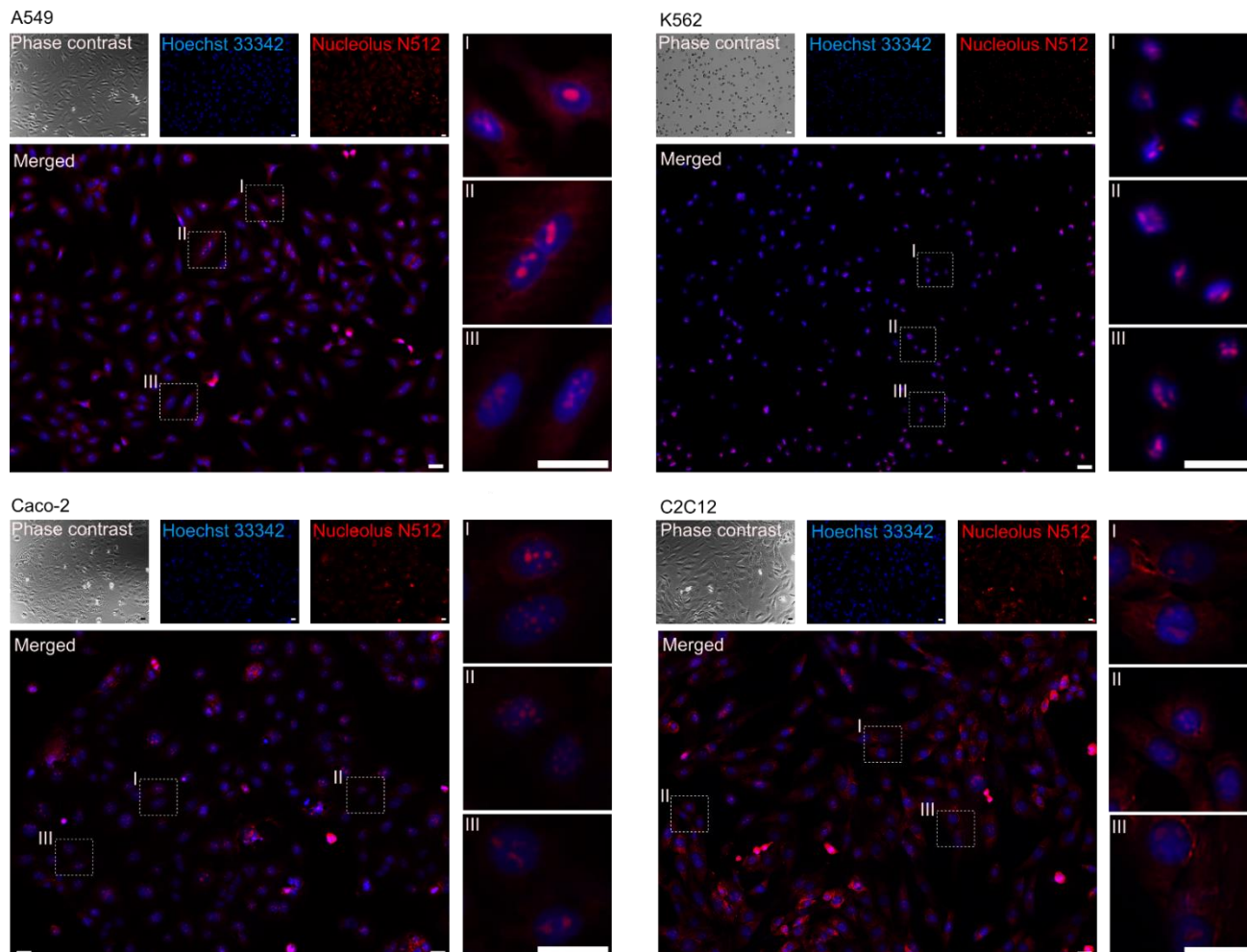


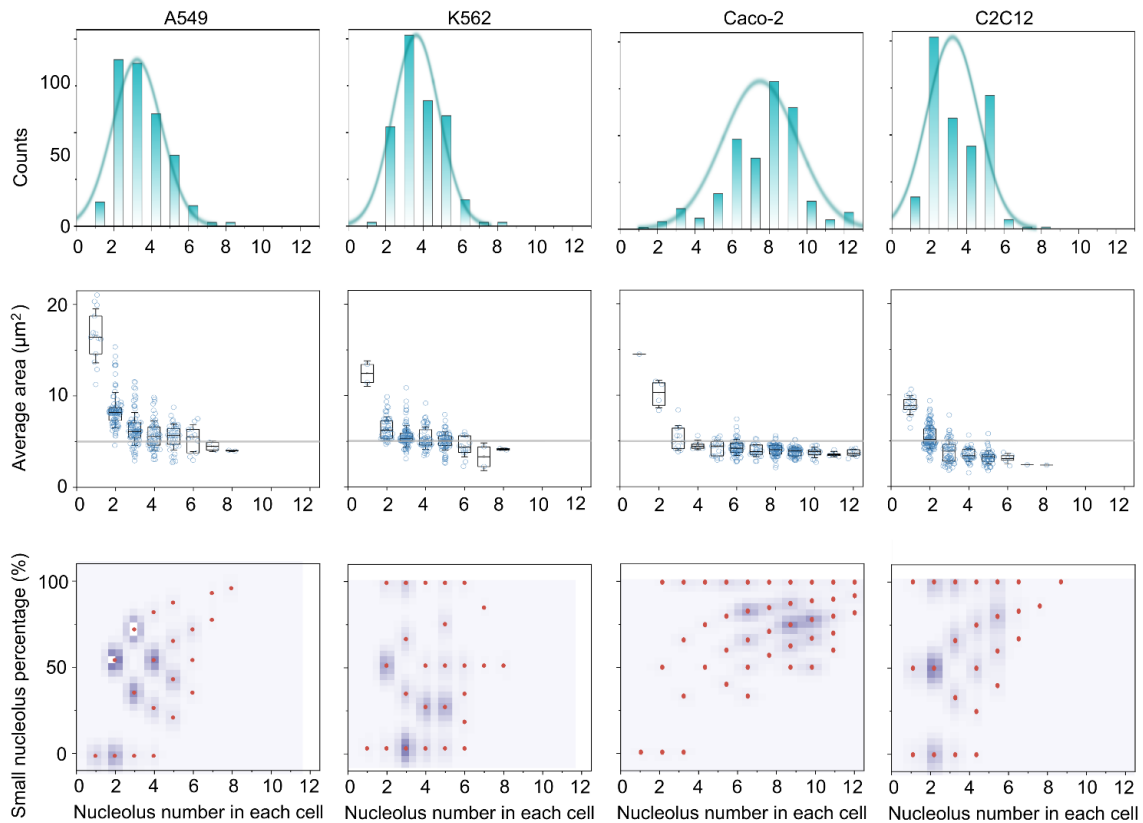
Fig.1 | Concept of the SLEC for label-free nucleolus analysis. **a**, Experiment setup of the scanning-based SLEC. The cells were sandwiched within a mirror-formed FP cavity. The upper mirror was spin-coated with a dye-doped PS layer, functioning as the gain medium. By adjusting the pump energy, lasing emissions can be achieved only within the nucleolus regions. **b**, Laser threshold and emitting spectrum in regions with and without nucleolus. **c**, Co-localization of the laser emission image with phase contrast image and fluorescence image from nucleolus dye. A549 cells were cultured on the lower mirror as an illustrative example. Scale bar: 5 μm . **d**, Enlarged comparison among phase contrast image, laser emission image and fluorescence image. Scale bar: 5 μm . The lasing emission image indicated distinct subareas attributed to the nucleolus' inhomogeneity of the refractive index (red arrows indicated). **e**, Configuration of the scanning-based SLEC for tissue sections. **f**, Configuration of the flow-based SLEC for suspended cells. Phase contrast image of a live K562 cell (20X objective), the nucleolus laser emission hyperspectral image and its spectrum. Scale bar: 5 μm . Each nucleolus corresponds to its unique laser peak.

Some other major changes in figures:

We added validation by fluorescence imaging method in Supplementary Fig. S19 and Supplementary Fig. S20.

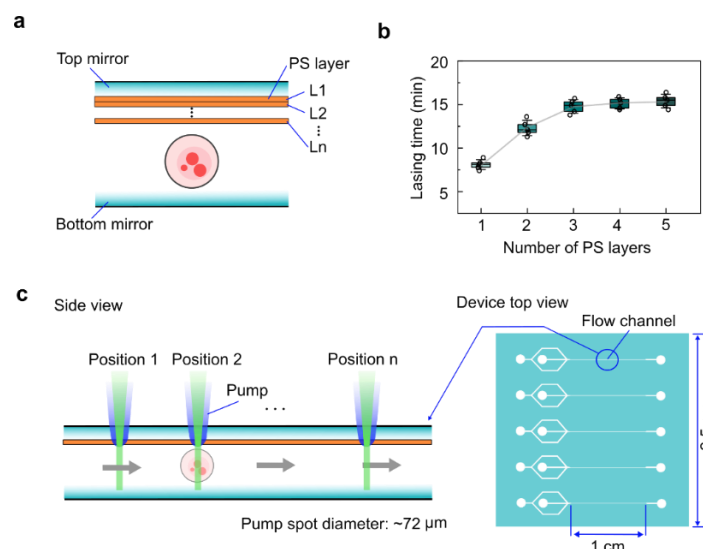


Supplementary Fig. S19. Images of the nucleolus in A549, K562, Caco-2 and C2C12 cells. The cells were stained with commercial nucleolus dye (Nucleolus Bright Red, N512) and nuclear dye (Hoechst 33342). Scale bar: 20 μm .



Supplementary Fig. S20. Nucleolus characteristics from fluorescence image-based analysis. Nucleolus number, average area of the nucleolus, and percentage of small nucleolus were statistically investigated. (mean $\pm$ SD, N=302 for A549 cells, 301 for K562 cells, 300 for Caco-2 cells, and 300 for C2C12 cells).

We added Supplementary Fig. S22 to address the photobleaching issues.



Supplementary Fig. S22. Enhancement of the lasing duration. (a-b) Multiple PS layers spin-coated on the top mirror. Stable lasing time of single PS layer tested via microbeads was 8.2 mins.

With increasing the PS layers to 5, the stable lasing duration gradually increased to 15.4 mins. (c) Multiple pumping positions along the channel. By alternating pumping positions along the channel, we could maximize the lasing duration. With a pump spot diameter of $\sim 72\ \mu\text{m}$ under a 10x objective and a designed effective channel length of 1 cm, each flow channel comprises approximately 138 pump positions. Considering the previously discussed stable lasing time of 15 mins, the total stable lasing time per channel can approximate 2,070 mins. Furthermore, for a single mirror measuring $2.5\ \text{cm} \times 2.5\ \text{cm}$, 5 flow channels can be designed. Consequently, for a single device, the maximum achievable lasing time can reach 10,350 mins.

Fig. 3, Fig. 4 and Supplementary Fig. S12 were also revised.

The abstract was revised to highlight the novelty and tissue application.

Nucleolus, a recognized biomolecular condensate, serves as the hub for ribosome biogenesis within cell nucleus. Its quantity and morphology are discernible indicators of cellular functional states. However, precise identification and quantification of nucleolus remains challenging without specific labeling, particularly for suspended cells, [tissue-level analysis](#) and high-throughput applications. Here we introduce a single-cell laser emitting cytometry (SLEC) for label-free nucleolus differentiation through light-matter interactions within a Fabry-Perot photonic resonator. [The separated gain medium enhances the threshold difference by 36-fold between nucleolus and its surroundings, enabling selective laser emissions at nucleolar area while maintaining lower-order mode.](#) The laser emission image provides insights into structural inhomogeneity, temporal fluid-like dynamics, [and pathological application](#). Lasing spectral fingerprint depicts the quantity and size of nucleoli within a single cell, showcasing the label-free flow cytometry for nucleolus. This approach holds promise for nucleolus-guided cell screening and drug evaluation, advancing the study of diseases such as cancer and neurodegenerative disorders.

The introduction has been revised to pointing the limitations of probe-labeled techniques and the current constraints of laser emission techniques, while emphasizing the novelty and breakthrough of our method.

For instance,

[‘Probe-labeled techniques offer a high-level specificity for nucleolus targeting, which requires either particular labeling chemistry \(dye, specific antibodies, etc.\) or expression of fluorescence-tagged proteins for direct detection. However, the artificial probes make it challenging to study the native cellular interactions and processes. Furthermore, fluorescence-based techniques often contend with strong background noise.’](#)

[‘However, most laser emissions still fall short in furnishing essential bio-information concerning individual cells and subcellular details. One main reason is the simultaneous excitation of high-order modes along with low-order modes, that usually masks the effective information.’](#)

‘Due to higher refractive index, the nucleolus functions as an optical lens, resulting in a notably reduced lasing threshold compared to its surrounding subcellular components (Fig. 1b). Additionally, the separation of gain medium enhanced the threshold difference by 36-fold relative to the gain medium-labeled cells²⁴, effectively circumventing emissions with high-order modes (Supplementary Note 1).’

‘Importantly, our approach also enabled nucleolus laser emission from tissue sections, facilitating label-free pathological analysis (Fig. 1e). Furthermore, it allowed the laser emission from suspension cells, where identifying nucleoli using phase contrast imaging alone is challenging. Consequently, we developed the label-free laser-emitting flow cytometry (Fig. 1f). The laser spectral fingerprints can be correlated with the number and size of nucleoli within a single cell, owing to the elimination of high-order modes by the gain separation.’

The discussion part was revised. System updating to enhance the throughput was added in the section of Materials and Methods.

Point-by-point response

Reviewer #1 (Remarks to the Author):

[Comment]

The article described a single cell lasing cytometry for cell nucleolus profiling. The fundamental mechanism is the “focusing effect” by nucleoli due to their higher refractive index effectively creating a lens within nucleus. By sandwiching the cells within a Fabry-Perot resonator with a fluorescent thin layer as gain media, the “focusing effect” reduced the lasing threshold to allow significant emission from the Fabry-Perot resonator compared with non-nucleoli region. The fundamental mechanism is considered sound, with numerical simulation and extensive demonstrations in spreading cells, as well as suspended cells. The article also demonstrated flow cytometry that quantifies the emission spectrum, which was further used for cell type classification based on profiling. While the technical content is strong and the study is considered rigorously performed, there are concerns whether the technique would make significant impact in the form of flow cytometry, for the following reasons/comments.

[Response]

We would like to thank the reviewer’s constructive comments and are delighted that the reviewer considered this strong and rigorously performed.

About the concerns regarding the impact on flow cytometry: We assert the substantial significance of our work within the field of flow cytometry, supported by several key points:

- 1) **Label-free laser emission flow cytometry.** Our study marks the pioneering introduction of label-free laser emission flow cytometry. Laser emissions offer distinctive advantages over fluorescence-based techniques, including ultrahigh-contrast signal, ultrasensitive to subtle changes and unique laser spectrum information. These allow laser emission widely applications in biomedical engineering.

The advent of the first single-cell biological laser (Gather, M., et al., Nature Photon., 2011, 5, 406-410), sparked anticipation for its potential application in flow cytometry. In their work, Gather et al. envisioned the adaptation of single-cell lasing for flow cytometry or microfluidics, highlighting the directional, bright, and nanosecond pulsed emission's potential to enhance throughput and speed of analysis, along with the narrowband laser emission's potential for dense wavelength multiplexing. By the conclusion of 2023, Seok-Hyun Yun et al. achieved a milestone with the development of multi-pass laser emission flow cytometry through the attachment of microlasers to cells (Sheldon J. J. Kwok, et al., Nature Biomedical Engineering, 2024, 8, 310-324), affirming the unique advantages of laser emissions in flow cytometry.

In contrast to prior studies, our research represents the inaugural achievement of label-free laser emission flow cytometry. This breakthrough circumvents the need for complex staining procedures, enabling the investigation of cellular bioactivities in their native states.

- 2) **Pioneering flow cytometry targeting nucleoli.** Our study also introduces the first label-free flow cytometry method targeting nucleoli, an area of increasing scientific and clinical interest

due to their roles as indicators of cellular stress, models of liquid-liquid phase separation, and biomarkers for various diseases. Conventional fluorescence-based nucleolar assays often suffer from background fluorescence due to interactions with cellular RNA, posing challenges for flow cytometry application. While previous label-free cytometry methods have been proposed (Thomas Blasi, et al., Nature Communications, 2016, 7, 10256), they lacked specific nucleolar features such as size and quantity. Our approach fills this gap by providing comprehensive label-free flow cytometry targeting nucleoli, offering insights into nucleolar quantity, size, and density.

- 3) **High-quality signals in flow cytometry.** Our methodology provides high-quality signals in flow cytometry, leveraging the ultrahigh-contrast and unique laser spectrum characteristics of laser emissions. In contrast to conventional fluorescence signals, laser emissions offer minimal background interference, enhancing signal clarity and facilitating precise analysis.
- 4) **Laser emission imaging.** Our technique links the laser emission imaging with specific cellular structures. Previous investigations were hampered by cell staining or labeling, resulting in laser images devoid of discernible cellular features.

Hence, we contend that our study introduces novel methodologies and concepts in both the realms of flow cytometry and microlasers, which will make significant impact.

We have addressed all the other issues and concerns raised.

[Comment]

1. What is the throughput for the proposed cell cytometry? The camera exposure was 10ms exposure, and that is consider very slow in comparison to conventional flow cytometry.

[Response]

It is important to note that the throughput of our proposed cytometry is mainly determined by three key parameters: the repetition rate of the pulsed laser, the integration time of the spectrometer (for spectrum-based cytometry), and the exposure time of the camera (for image-based cytometry), as shown in Fig. R1. Therefore, throughput enhancement can be achieved through optimization of these parameters.

In the original manuscript, as the proof of concept, we demonstrated a throughput of ~ 25 events per second (eps), constrained by the repetition rate of the pulsed laser we utilized (50 Hz, EKSPLA NT230). We acknowledge that this throughput is relatively slow compared to imaging flow cytometry (100-2,000 eps) and conventional cytometry (FACS) (1,000-10,000 eps) (Gregor Holzner, et al., Cell Reports, 2021, 34, 108824; Nao Nitta, et al., Cell, 2018, 175, 266-276).

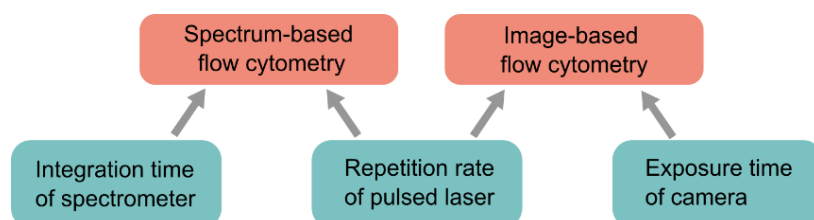


Fig. R1 Key parameters of the two types of cytometry.

To enhance the throughput of the laser emitter cytometry, we made significant system updates. We replaced the pulsed laser with a high repetition rate laser $\sim 5\text{kHz}$ (Microlaser SB1-473-3-5, BrightMicrolaser Ltd., Italy). The spectrometer was upgraded to a faster and tunable integration time $2\mu\text{s}$ -20s (0.05-500kHz, AvaSpec-ULS2048XL-EVO, AVANTES Ltd., Netherlands). Additionally, the camera (FASTCAM Mini UX50, Photron) was capable of a frame rate of 2500 fps. With these improvements, we achieved a throughput of $\sim 2,000$ eps for spectrum-based cytometry and $\sim 1,000$ eps for image-based cytometry, comparable to commercial cytometry systems.

Further enhancements are possible with updates to the pulsed laser, spectrometer, and high-speed camera. For example, a pulsed laser with a repetition rate of up to 100kHz (BrightMicrolaser Ltd., Italy) could theoretically enable spectrum-based cytometry to achieve a throughput of 50,000 eps. Similarly, a camera with a frame rate of 6,250 fps (FASTCAM Mini UX100, Photron) could theoretically enhance image-based cytometry to approximately 3,125 eps. These advancements illustrate the potential for further improvement in throughput.

[Changes made]

We have added the description about throughput and discussed it in Materials and Methods of the manuscript as following:

“To further increase the throughput, we replaced the pulsed laser with a high repetition rate laser

operating at 5kHz (Microlaser SB1-473-3-5, BrightMicrolaser Ltd., Italy). Additionally, we replaced the spectrometer with a faster one featuring a tunable integration time of 2 μ s-20s (0.05-500kHz, AvaSpec-ULS2048XL-EVO, AVANTES Ltd., Netherlands). The flow rate was increased to ~ 60 mm s⁻¹. As a result of these enhancements, the throughput was boosted to 2000 eps.”

[Comment]

2. The photobleaching for the fluorescence polystyrene is a significant limitation for the practical use of the device (<5 mins total exposure to avoid photobleaching). Unless there is a way to recharge the fluorescence layer, it is hard to envision a prolong use for the device.

[Response]

Thanks for the reviewer's comments. We acknowledge that addressing the issue of photobleaching in the dye-doped PS layer presents a significant challenge for the functionality of this device. Here, we propose two solutions to mitigate this challenge and two potential solutions to solve this challenge.

1. **Multiple PS layers on top mirrors to enhance the lasing duration** (Fig. R2a). By spin-coating multilayers, the gain medium can be increased. The experimental data presented in the original manuscript pertains to a single PS layer formed through a single spin-coating, with a thickness of $\sim 1 \mu\text{m}$ (see Materials and Methods Section). Tests utilizing $5 \mu\text{m}$ silicon beads revealed a stable lasing time of ~ 8.2 mins under a pump energy of $68.3 \mu\text{J mm}^{-2}$. Upon spin-coating an additional PS layer onto the top mirror, the lasing time extended to 12.3 mins. The lasing duration reached 15.4 mins with 5 layers spin-coated on the top mirror (Fig. R2b). The thickness of 5 PS layers was about $4.2 \mu\text{m}$. Thus, by increasing the thickness of the PS layer, the stable lasing time can be extended to about 15 mins.

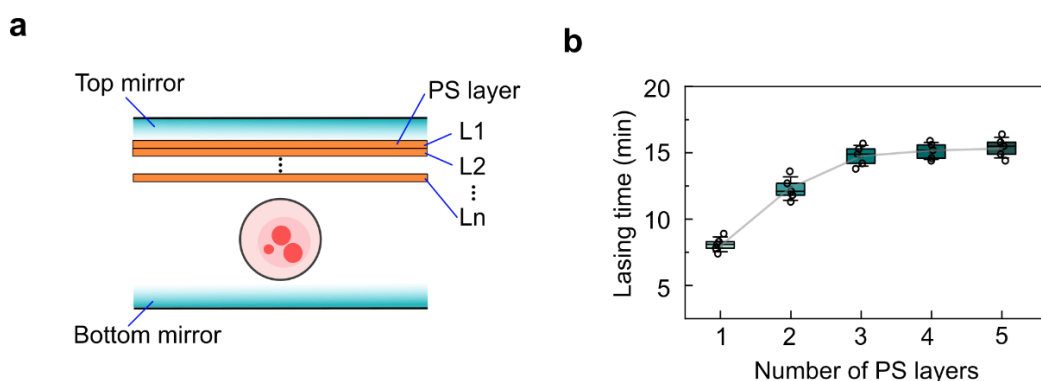


Fig. R2 Multiple PS layers to enhance the lasing duration. (a) Adding the number of PS layer via multiple spin-coating. (b) Relationship of stable lasing time and PS layer number on the top mirror.

2. **Multiple pumping positions along the channel.** Although only one position within the flow channel was initially pumped, we explored the possibility of pumping various positions along the channel. Given that cells flow throughout the entire channel, all areas within it can be utilized. Therefore, by alternating pumping positions along the channel, we could maximize the lasing duration (Fig. R3). With a pump spot diameter of $\sim 72 \mu\text{m}$ under a 10x objective and a designed effective channel length of 1 cm, each flow channel comprises approximately 138 pump positions. Considering the previously discussed stable lasing time of 15 mins, the total stable lasing time per channel can approximate 2,070 mins. Furthermore, for a single mirror measuring $2.5 \text{ cm} \times 2.5 \text{ cm}$, 5 flow channels can be designed (Fig. R3). Consequently, for a single device, the maximum achievable lasing time can reach 10,350 minutes (Table R1). This duration is deemed sufficient for typical flow cytometry applications.

Additionally, it is worth noting that the top mirror can be readily replaced with a new mirror, and

both the mirror and PS layer can be economically batch-fabricated.

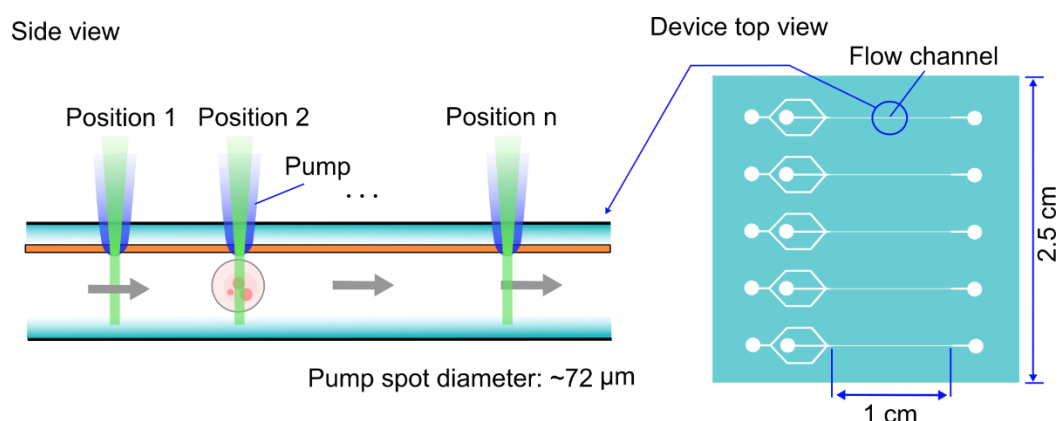


Fig. R3 Multiple pumping positions along the channel. Multi-flow channels can be further designed on the device.

Table R1 Parameters of multiple pumping positions

Pump spot diameter (μm)	Effective length of flow channel (cm)	Pump position number on one channel	Lasing time for one position (min)	Flow channel number on the device	Effective working time per device (min)
72	1	138	15	5	10,350

Two potential solutions to solve this challenge:

1. **Implementation of high-efficiency gain medium.** Presently, the doped gain medium utilized is Bodipy-1, an organic dye. To enhance efficiency and mitigate photobleaching, more effective gain media can be explored (Fig. R4a). Promising alternatives include perovskite materials (Chuanjiang Qin, et al., Nature, 2020, 585, 53-57), rare-earth-doped nanoparticles (Angel Fernandez-Bravo, et al., Nature Materials, 2019, 18, 11), carbon dots (Antonion Madonia, et al., ACS Nano, 2023, 17, 21274-21286), and even organic semiconductor films (Markus Karl, et al., Nature Communications, 2018, 9:1525).
2. **Laminar flow for continuous dye charging.** Introducing the dye solution into the flow channel alongside the cell medium allows for the formation of a thin flowing film utilizing the laminar flow mechanism (Fig. R4b). This approach presents a promising solution to address the photobleaching issue effectively.

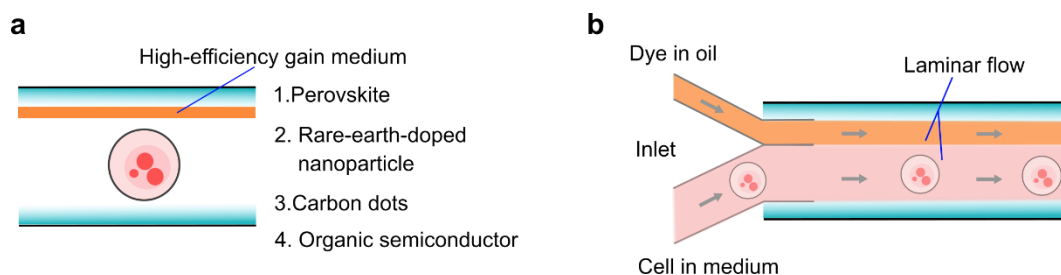


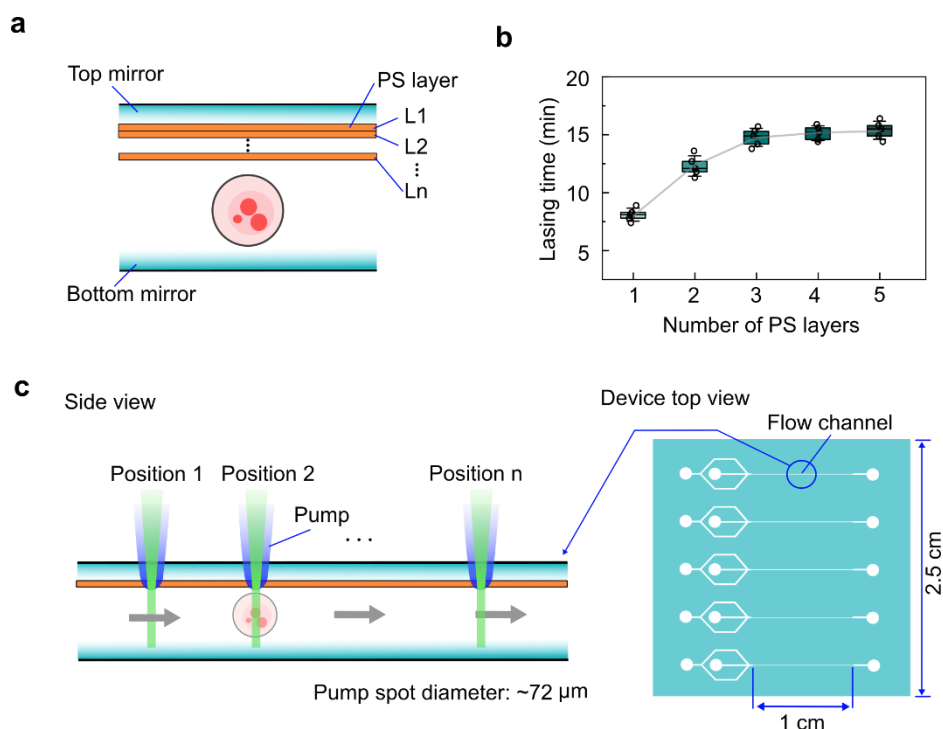
Fig. R4 Two potential solutions to the photobleaching. (a) High-efficiency gain medium for prolonged lasing time. (b) Laminar flow for continuous dye layer charging.

[Changes made]

We have added the following sentences into the revised manuscript.

“To mitigate the impact of dye photobleaching, each area for single PS layer was pumped for 5 mins. To enhance the lasing duration, multi-PS layers and multiple pumping positions along the channel were investigated. The maximum achievable lasing time can reach 10,350 mins (Fig. S22).”

We have added the description of the former two methods in SI information as below:



Supplementary Fig. S22. Enhancement of the lasing duration. (a-b) Multiple PS layers spin-coated on the top mirror. Stable lasing time of single PS layer tested via microbeads was 8.2 mins. With increasing the PS layers to 5, the stable lasing duration gradually increased to 15.4 mins. (c) Multiple pumping positions along the channel. By alternating pumping positions along the channel, we could maximize the lasing duration. With a pump spot diameter of $\sim 72 \mu\text{m}$ under a 10x objective and a designed effective channel length of 1 cm, each flow channel comprises approximately 138 pump positions. Considering the previously discussed stable lasing time of 15 mins, the total stable lasing time per channel can approximate 2,070 mins. Furthermore, for a single mirror measuring $2.5 \text{ cm} \times 2.5 \text{ cm}$, 5 flow channels can be designed. Consequently, for a single device, the maximum achievable lasing time can reach 10,350 mins.

[Comment]

3. The multiple laser peaks from larger nucleoli is reasoned to be HRIUs (high refractive index units). It seems more like lasing with multimode?

[Response]

We acknowledged the reviewer's comments.

To ascertain whether the observed laser peaks and the laser emission pattern originate from a single mode or high-order multimode, we utilized a modified spectrometer to image the laser emissions. This setup allows for the spatial imaging of laser emissions based on their spectral components through diffraction gratings. This process generated a hyperspectral image of the laser output, captured on a camera (Gather, M., et al., Nature Photon., 2011, 5, 406-410). Here we demonstrated the hyperspectral images from two large nucleoli (Fig. R5a) under a pump energy of $\sim 68.3 \mu\text{J mm}^{-2}$. Statistical analysis revealed that most laser peaks in large nucleoli emanate from the [0,0] single mode, with only a minority representing higher-order multimode emissions, such as [1,1].

Furthermore, the laser imaging displayed strong congruence with the HRIUs as indicated by the phase contrast images. For example, Fig. R5b shows a large nucleolus with distinct internal shading, suggestive of the positions of HRIUs. The corresponding laser emission closely aligned with these HRIUs. It is important to note that the visibility of HRIUs under phase contrast microscopy, as clear as shown in Figure R5b, is not typical; their appearance can often be less pronounced.

In addition, theoretically, the separation of gain could elevate the threshold and mitigate emissions associated with high-order modes (See detailed discussion in Supplementary Note 1).

In conclusion, for large nucleoli, the laser emission imaging is well-aligned with the HRIUs, and the associated laser peaks predominantly arise from the single mode rather than from multimode.

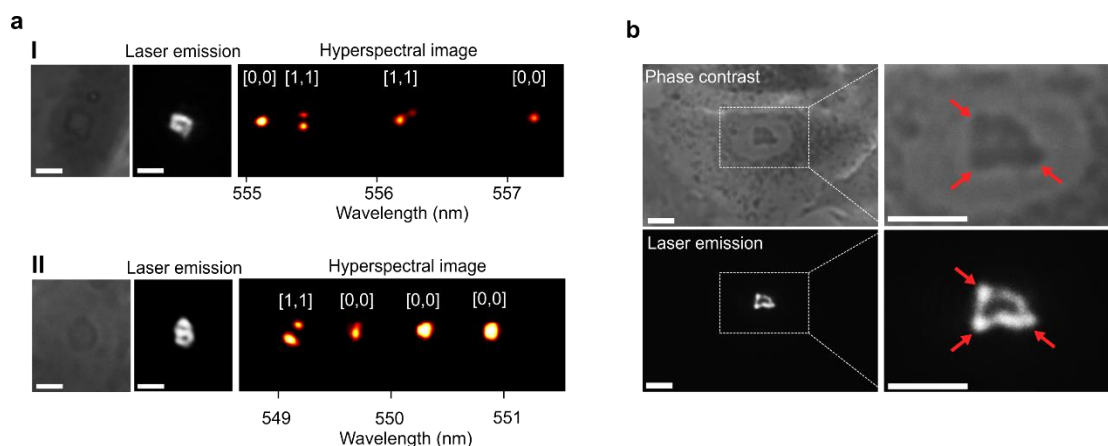


Fig. R5 Characterization of lasing emission in large nucleoli. (a) Far-field laser emission images and hyperspectral images of the lasing modes from large nucleoli. Scale bar: 5 μm . (b) A large nucleolus that showed various shades under phase contrast image. The laser image showed good alignment with the HRIUs indicated by phase contrast image. Scale bar: 5 μm .

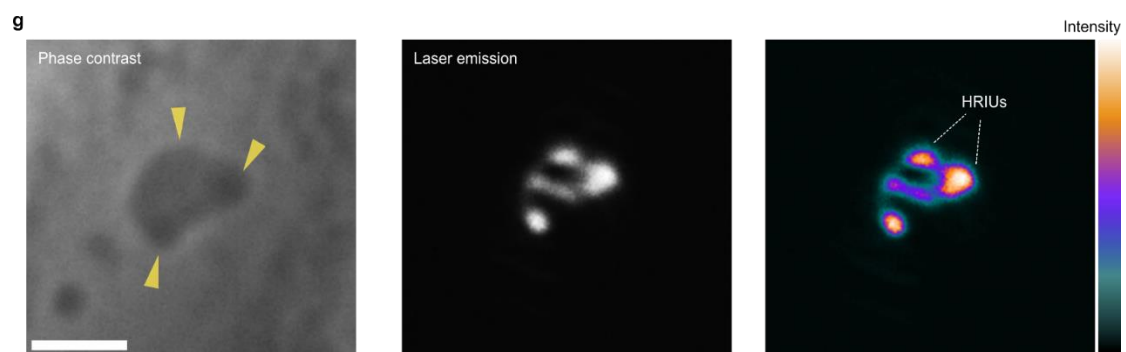
[Changes made]

Followed the suggestion, we have revised the manuscript as below:

“To determine whether the laser emission patterns and spectrum stem from single mode or higher-order modes, we employed a modified spectrometer to image the laser emissions. This setup enabled the laser emission to be spatially imaged according to spectral components through diffraction gratings, resulting in a hyperspectral image of the laser output on the camera. Figure 3f-g compares the laser emission images to the hyperspectral image. The results indicate that the laser emission image for small nucleoli predominantly emanates from a single transverse laser mode, labeled as the [0,0] mode (Fig. S8). In larger nucleoli, the laser emission image primarily derives from the [0,0] single modes, but higher-order modes, such as [1,1] modes, become more. In theory, the separation of gain medium enabled the primary emissions without high-order modes (See supplementary Note1)..... Thus, in general, the majority of laser spectral peaks within an FSR correspond to the HRIUs.”

“The laser emission image also showed well-aligned with the HRIUs (Fig. S7).”

We revised the figures in SI information as below:



(g) A large nucleolus that exhibited various shades inside under phase contrast image. The laser emission image showed good alignment with the HRIUs indicated by phase contrast image. Scale bar: 5 μm .

[Comment]

4. The approach is specifically developed to profiling nucleoli. While the case is made in the label-free approach without introducing fluorescent target, the advantage is offset by the exclusive design only for nucleoli. I found it less compelling with more conventional imaging based cytometry, using live cell nucleoli dyes (e.g. <https://www.enzolifesciences.com/ENZ-51009/nucleolar-id-green-detection-kit/>).

[Response]

Thanks for the reviewer's comments. In previous studies (Gather, M., et al., Nature Photon., 2011, 5, 406-410; Matjaž Humar, et al., Opt. Express., 2015, 23, 21), the laser emission is homogeneous across the entire cell, posing challenges for reflecting specific cellular structures via laser emissions. We respectfully disagree with the comments and believe our method, which strategically enables selective laser emission in the nucleolar area, represents a revolutionary improvement for nucleolus study and microlaser applications.

In addition, the nucleolus, as the largest organelle within the nucleus, plays a pivotal role in coordinating ribosome biogenesis and RNA editing (Karan J. Abraham, et al., Nature, 2020, 585, 298-302; Dubois ML., et al., The Functional Nucleus, Springer, Cham., 2016). Its importance has been increasingly recognized due to several key functions:

1. **Indicator of cellular and nucleolar stress:** The nucleolus serves as a natural indicator for cellular and nucleolar stress, responding to various stress factors such as hypoxia, protein misfolding, DNA damage, and oxidative stress (Shaqraa Musawi, et al., Nature Communications, 2023, 14, 7384; Xiaocen Jin, et al., Nature Communications, 2023, 14, 9; Kai Yang, et al., Nature Communications, 2016, 7, 13599). Investigating stress responses through nucleolar sensors contributes to understanding biological mechanisms underlying aging, cancers, and neurodegenerative diseases (Jana Riegger, et al., Cellular & Molecular Biology Letters, 2023, 28:76).
2. **Good model for liquid-liquid phase separation (LLPS):** The nucleolus provides an excellent model for studying liquid-liquid phase separation (LLPS) in cellular environments, a phenomenon crucial for the formation of membraneless organelles and implicated in various human diseases (Mitrea D.M. et al., Nature Communications, 2018, 9, 842; Lafontaine D.L.J., Nature Reviews Molecular Cell Biology, 2021, 22, 165-182; Joshua A. Riback, et al., Nature, 2020, 581, 209-214).
3. **Biomarkers for disease:** Alterations in nucleolar morphology serve as biomarkers for diseases such as cancer and neural disorders. For example, nucleolar size and quantity variations are associated with prognosis in various tumors and represent cellular hallmarks of longevity and metabolic health (Tiku V., et al., Nature Communications, 2017, 18, 651; Zlotorynski, et al., Nature Reviews Molecular Cell Biology, 2018, 28, 662-672; Hein N., et al., Trends in Molecular Medicine, 2013, 19, 643-654).

Given these considerations, developing tools that specifically target nucleolar studies holds significant scientific and clinical relevance.

Our method presents obvious advances over conventional imaging techniques that utilize nucleolar dyes, making it particularly suited for in-depth cellular studies:

1. **Label-free imaging in natural state:** Our approach enables label-free imaging, allowing for the investigation of nucleolar activities in their natural state. By studying native molecules, label-free techniques provide insights into cellular interactions without introducing artificial probes or labels, thereby reflecting natural molecular interactions and cellular processes (Lisa-Maria Needham, et al., Nature, 2024, doi: 10.1038/s41586-024-07370-8; Voula Kodoyianni, BioTechniques, 2018, 50, 1). This is particularly crucial for analyzing cellular stress and LLPS studies within the nucleolus. In addition, it avoids the complex staining processes, thus streamlining the analysis of patient-derived samples.
2. **Ultrahigh-contrast imaging with minimal background:** Compared to fluorescence imaging, laser emission offers ultrahigh contrast imaging, enabling detailed examination of nucleolar structures (as depicted in Fig. 1d in the manuscript). In contrast, conventional nucleolar dyes, which often react with RNAs, can lead to strong fluorescence background due to RNA presence in the cytoplasm, impairing precise analysis and potentially complicating its use in flow cytometry (<https://dojindo.com/ASIA/products/N512>; <https://www.enzolifesciences.com/ENZ-51009/nucleolar-id-green-detection-kit/>).
3. **Unique laser spectrum for comprehensive analysis:** Unlike fluorescence imaging, laser emission also delivers a unique spectrum that can elucidate multiple parameters of the nucleolus, such as number, size, and density.

In summary, the proposed label-free method is a valuable tool for nucleolar analysis, with significant potential for advancing the study of diseases such as cancer and neurodegenerative disorders and enhancing nucleolus-guided cell screening and drug evaluation.

[Changes made]

Following the suggestion, we have revised the following sentences in the manuscript:

The significance of nucleolus study has been emphasized in Paragraph 1 in background.

“Nucleolus is the most paramount structure in the nucleus of eukaryotic cells. It takes the form of a spherical biomolecule condensate encompassing proteins, RNA, and DNA, coordinating ribosome biogenesis and RNA editing [emphasizing function]. This membraneless organelle has garnered increased attention with the liquid-liquid phase separation emerging as a new paradigm. Leveraging its fluid-like dynamics, the nucleolus adeptly adjusts its dimensions, count, and protein composition in response to shifts in cellular homeostasis [emphasizing models in LLPS]. Consequently, the nucleolus has evolved into a potent biomarker for disease and a sensitive biosensor for cellular stress. For instance, diminutive nucleoli stand as a cellular hallmark of longevity and metabolic health conserved across taxa. Conversely, augmented nucleolar size and quantity are recognized as clinically pertinent indicators of adverse prognosis in numerous types of tumors. [emphasizing biomarker of disease]”

The advantages of label-free technique have been emphasized in Paragraph 2 in introduction.

“Probe-labeled techniques offer a high-level specificity for nucleolus targeting, which requires either

particular labeling chemistry (dye, specific antibodies, etc.) or expression of fluorescence-tagged proteins for direct detection. However, the artificial probes make it challenging to study the native cellular interactions and processes.”

The following words have been added into the discussion part to enhance the importance of laser emission technique and label-free analysis.

“Compared to fluorescence imaging, laser emission offers ultrahigh-contrast imaging with minimal background and unique spectrum information.”

“Label-free approach holds paramount importance in the study of subcellular organelles, as it provides a direct means to glean information, particularly concerning the natural kinetics of protein. Nucleolus, as a quintessential model of condensate organelles, exhibits a significant demand for label-free characterization.”

Reviewer #2

[Comment]

This paper reports the establishment of a method for the analysis of nucleoli using laser emission in a Fabry-Perot photonic resonator. This method enables nucleolus-selective lasing by taking advantage of the lower laser emission threshold derived from the higher refractive index of the nucleolus. The authors showed that this method of analyzing laser emission images and spectral fingerprints allows for a more accurate assessment of nucleolus number, morphology, fluid-like dynamics, and heterogeneity than the conventional methods. The development of detection methods for nucleolus state changes is important because they are used in the diagnosis of cancer pathology and as indicators of cellular stress. The advantage of being able to assess the quality and quantity of nucleoli in individual living cells without the need for specialized stains or the introduction of exogenous fluorescent proteins makes this technique an effective tool for drug evaluation and the study of diseases such as cancer and neurodegenerative disorders. In particular, analysis by flow cytometry has great potential. However, this method is not suitable for the detection of cells in tissues because it can only be applied to individually isolated cells such as cultured cells, making it difficult to use in histopathology, which is the most important and necessary application method. In this regard, the evaluation of this report must be lowered. Therefore, this paper is not recommended for publication in the Journal of Nature Communications.

[Response]

Thanks for the reviewer's comments. We appreciated that the reviewer considered this detection method is important and more accurate, which would be an effective tool for drug evaluation and the study of diseases.

[Response – tissue applications]

We appreciate the reviewer's insightful comment regarding the potential tissue-level applications of our technique. This is indeed a crucial aspect of our research, and in response, we have conducted experiments to validate the technique's applicability in tissue analysis.

Theoretically, our technique harnesses the unique property of nucleoli having a higher refractive index than their surrounding environment to achieve targeted laser emission. This principle is equally applicable at the tissue level, where nucleoli maintain their relatively higher refractive index. For tissue-level analysis, such as histopathology, the required tissue slice typically has a thickness of $\sim 10\ \mu\text{m}$, corresponding to roughly a single layer of cells. Our device configuration accommodates this, with the distance between mirrors ranging from 9.2 to 30 μm , which can effectively sandwich the tissue slice.

Unlike in phase contrast imaging where distinguishing nucleoli can be challenging, our technique successfully elicited clear laser emissions from the nucleolar regions (Fig. R6a). Statistical comparison of laser emissions between mice tumor tissues and healthy tissues revealed significant difference (Fig. R6b). In tumor tissues, there was a notably higher percentage of large lasing spots, indicating the presence of larger nucleoli. Additionally, the density of lasing spots was substantially higher in tumor tissues, and nucleoli in these tissues exhibited a lower circularity, suggesting altered nucleolar morphology associated with pathological states.

These results underscore the potential of our lasing technique as a powerful tool for tissue-level analysis, providing novel insights into tissue pathology that are not readily apparent through traditional imaging methods.

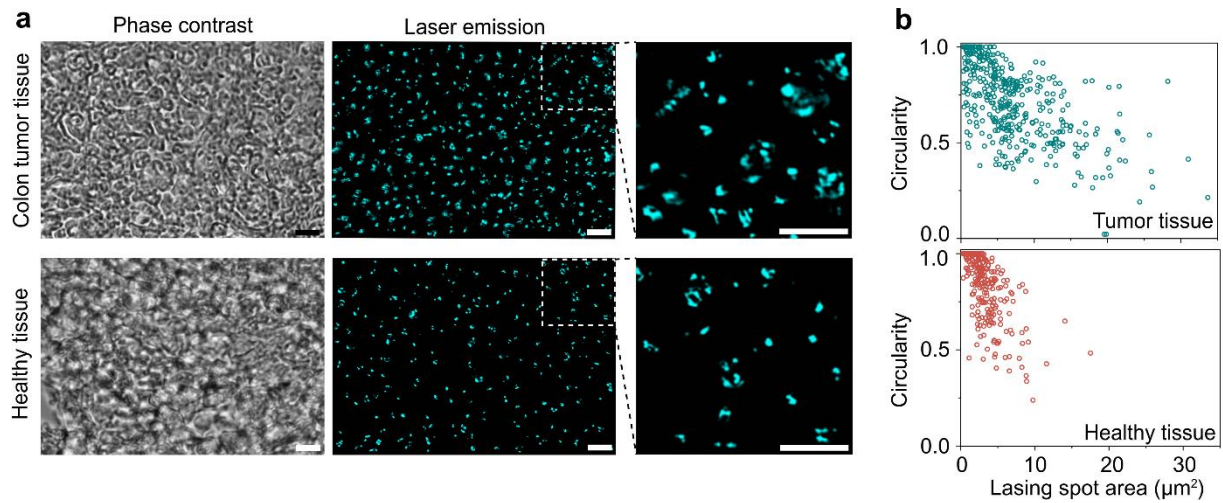


Fig. R6. Characterization of the lasing emission at the tissue level. (a) Lasing emission obtained from mice colon tumor tissue and healthy tissue. In tumor tissue, the lasing emissions exhibited enhanced area and density. Scale bar: 20 μm . (b) Statistical analysis of the lasing spot area and circularity, correlating these measurements with nucleolar morphology variations between tumor and healthy tissues.

The additional tissue samples have also undergone investigation and exhibited comparable nucleolar features (Fig. R7).

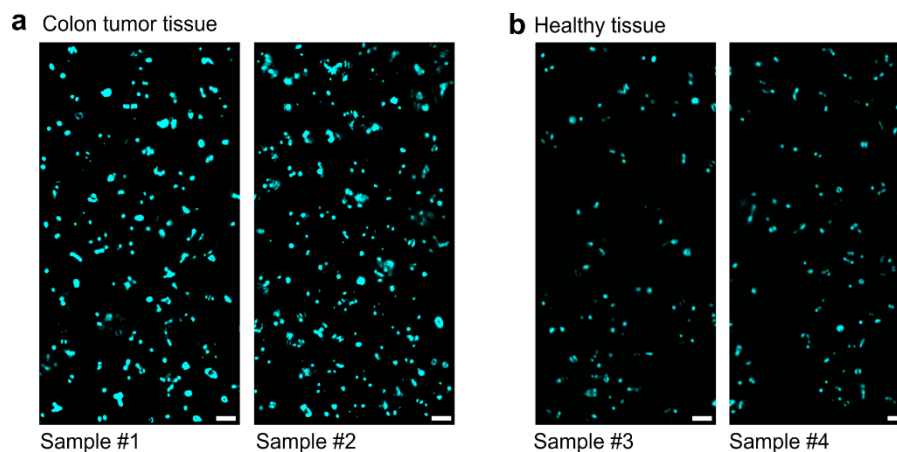


Fig. R7. Laser emission from the tissue samples. (a) Colon tumor tissues. (b) Healthy tissues. Scale bar: 10 μm .

To validate our results, we stained the tissues with HE (Fig. R8). In colon tumor tissue, conspicuous and enlarged nucleoli were observed, with some displaying altered morphology. Conversely, in healthy tissue, nucleoli were less apparent and exhibited smaller size. Notably, these findings mirror nucleolar features obtained through our technique. It is worth reiterating that our technique

circumvents the need for complex staining procedures, offering ultrahigh-contrast images compared to probe-labeled methods.

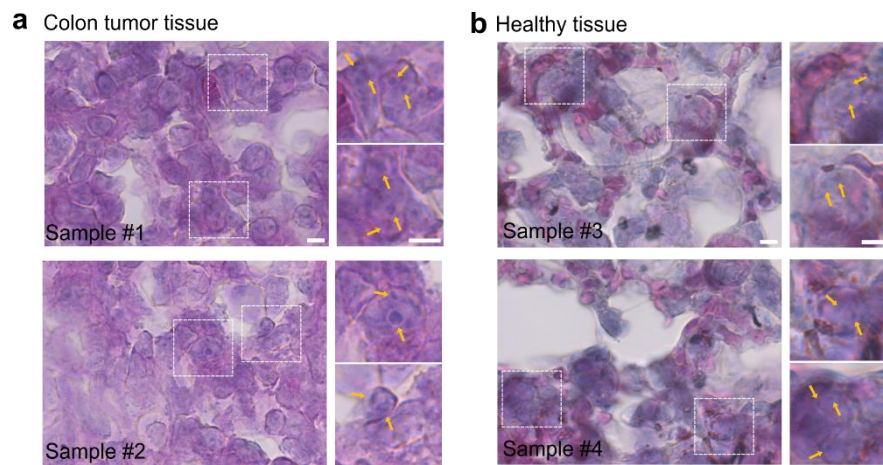


Fig. R8. Tissue sections with HE stains. (a) Colon tumor tissues. (b) Healthy tissues.

Scale bar: 10 μm .

[Changes made]

We added an additional section in the revised manuscript to investigate the laser-emission performance at the tissue level.

Laser emission from tissues

The nucleolus serves as a pivotal biomarker widely employed in histopathological analyses. we investigated label-free laser emissions at the tissue level. To facilitate comparison, slices of mice colon tumor and healthy tissues ($\sim 10\ \mu\text{m}$ thick each) were sandwiched between mirrors. Through adjustment of pump energy, we successfully induced laser emissions from these tissues (Fig. 5a). Contrary to phase contrast imaging, where distinguishing nucleoli can be challenging, laser emissions distinctly highlighted the nucleolar regions. In addition, this method works both fresh tissues and fixed tissues.

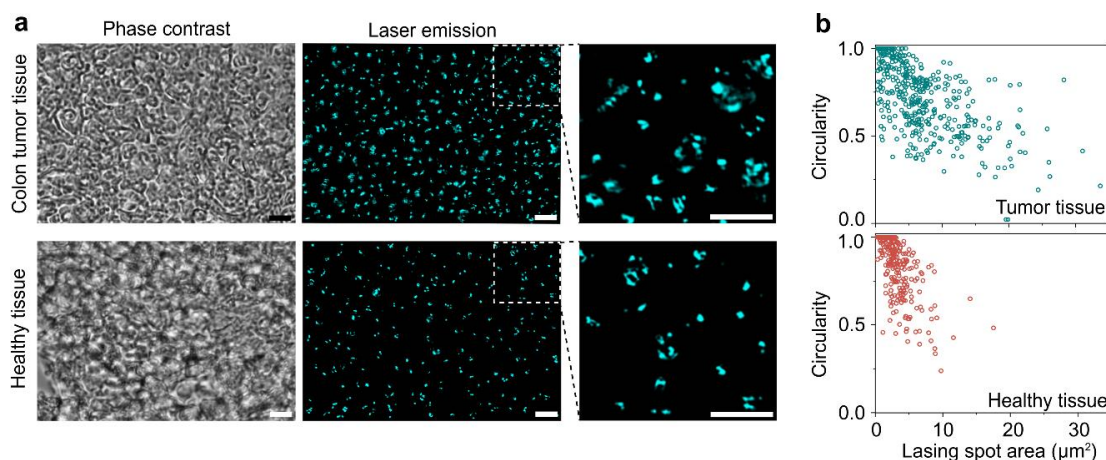
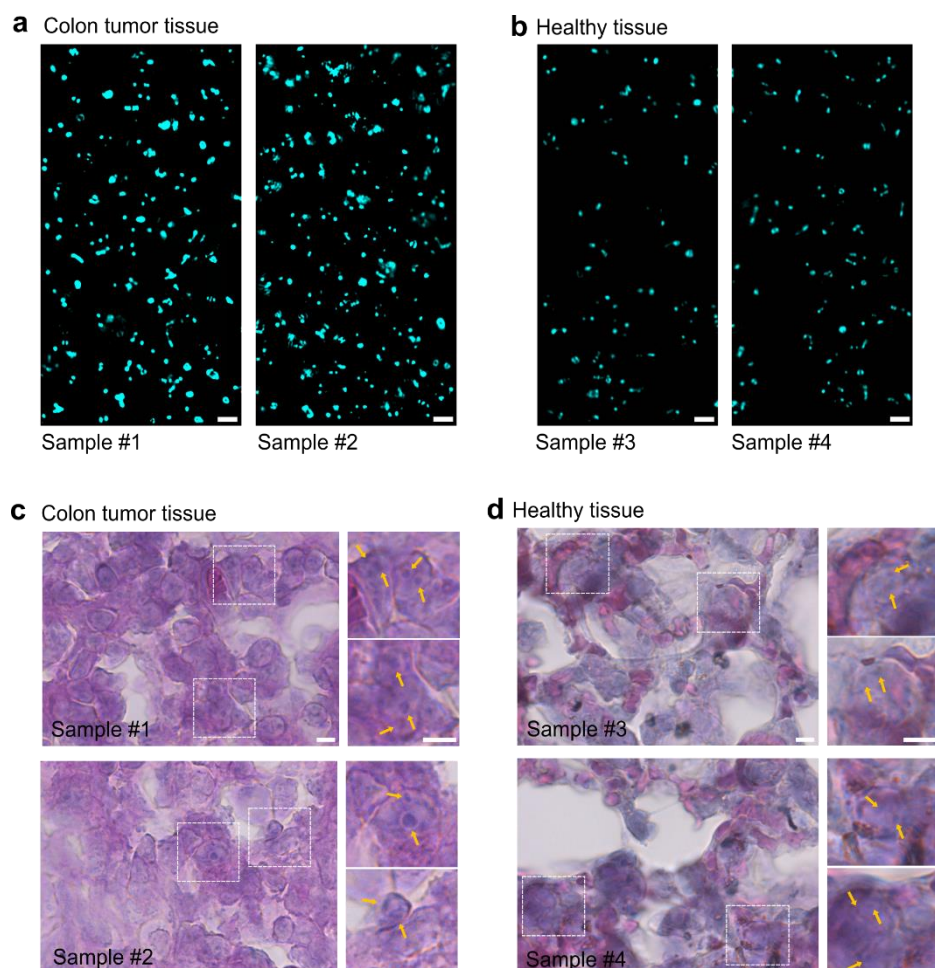


Fig. 5 | Characterization of the laser emission at the tissue level. **a**, Laser emission obtained from mice colon tumor tissue and healthy tissue. Scale bar: 20 μm . **b**, Statistical analysis of the lasing spot

area and circularity, correlating with nucleolar morphology variations between tumor and healthy tissues.

Statistical analysis of the laser emissions from mice tumor and healthy tissues demonstrated a significant difference (Fig. 5b). Notably, tumor tissues exhibited a higher proportion of large lasing spots, indicative of enlarged nucleoli. Furthermore, the density of lasing spots was significantly greater in tumor tissues, with nucleoli in these samples showing decreased circularity, indicative of altered nucleolar morphology associated with pathological conditions. Similar results were observed across multiple sample batches (Fig. S15a-b). To validate the results, we compared them with hematoxylin and eosin (HE) stained images (Fig. S15c-d). Our results exhibited similar nucleolar features achieved by HE stains. These underscore the potential of this technique as an effective tool for tissue-level analysis.

Laser emission from the additional samples and the comparison with HE stains were added in the SI as Supplementary Fig. S15.



Supplementary Fig. S15. Characterization of laser emission from tissues. (a-b) Laser emission from the additional tissue samples. Scale bar: 10 μ m. (c-d) Images of the tissues with HE stains. Scale bar: 10 μ m. Notably, in colon tumor tissue, conspicuous and enlarged nucleoli were observed,

some exhibiting altered morphology. Conversely, in healthy tissue, nucleoli were less prominent and displayed smaller size. This indicated that the laser emission technique could revealed similar nucleolar features with simple process and ultrahigh-contrast signals.

The following description was added in the Section of Materials and Methods.

Tissue experiment Tumors and healthy tissues for this study was from p53^{+/-} mice (initially from the Jackson lab). All the mice were bred and maintained under non-specific pathogen-free conditions at the animal research facility of Nanyang Technological University. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Tumors and healthy tissues were dissected and then fixed with 4% PFA overnight. They were immersed in 30% sucrose in PBS for three days, and then they were freshly frozen by cooling dry ice in methylbutane following by OCT-imbedding in -80°C. They were cut into 10 μ m section. The HE stained tissues were observed subject under a 100x objective by Zeiss microscope.

To highlight this part, Fig.1 has been revised.

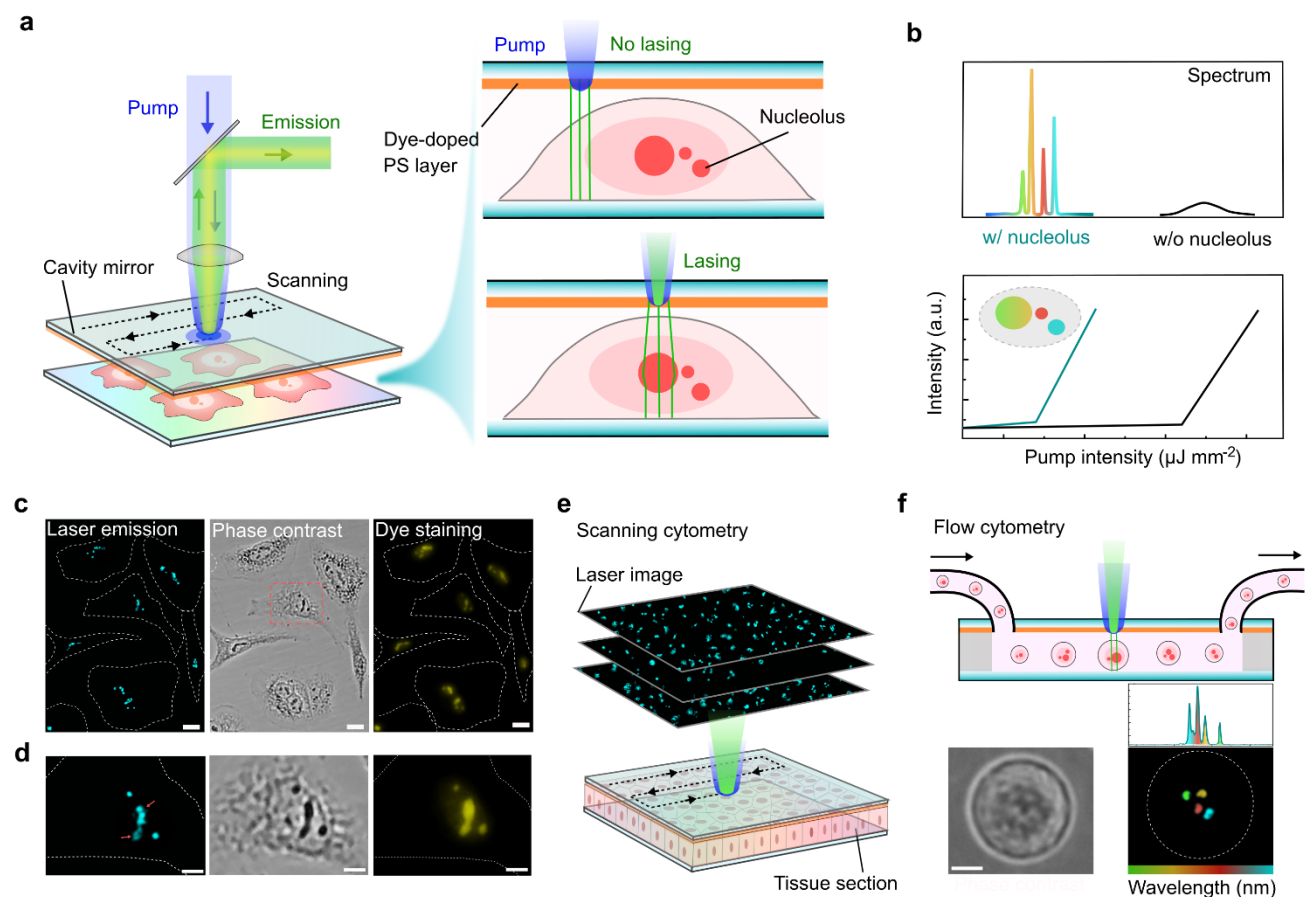


Fig.1 | Concept of the SLEC for label-free nucleolus analysis. **a**, Experiment setup of the scanning-based SLEC. The cells were sandwiched within a mirror-formed FP cavity. The upper mirror was spin-coated with a dye-doped PS layer, functioning as the gain medium. By adjusting the pump energy, lasing emissions can be achieved only within the nucleolus regions. **b**, Laser threshold and emitting spectrum in regions with and without nucleolus. **c**, Co-localization of the laser emission image with phase contrast image and fluorescence image from nucleolus dye. A549 cells were

cultured on the lower mirror as an illustrative example. Scale bar: 5 μm . **d**, Enlarged comparison among phase contrast image, laser emission image and fluorescence image. Scale bar: 5 μm . The laser emission image indicated distinct subareas attributed to the nucleolus' inhomogeneity of the refractive index (red arrows indicated). **e**, [Configuration of the scanning-based SLEC for tissue sections](#). **f**, Configuration of the flow-based SLEC for suspended cells. Phase contrast image of a live K562 cell (20X objective), the nucleolus laser emission hyperspectral image and its spectrum. Scale bar: 5 μm . Each nucleolus corresponds to its unique laser peak.

Specific comments:

[Comment]

1. Figure 4-f-II: What is the laser emission observed in the metaphase detecting? Is it related to the nucleolus or is it laser emission resulting from an increase in refractive index due to chromosome aggregation?

[Response]

Thanks for the reviewer's question. The laser emission observed in the metaphase is from the chromosome aggregation. During the mitosis, the nucleolus would disassemble. During metaphase, nucleolar proteins concentrate around the chromosomes along the cell's equator, forming a liquid-like perichromosomal region. At this stage, the equatorial plane of the cell exhibited the highest refractive index. Thus, we observed the laser emissions around the equatorial plane.

[Changes made]

We have revised the description of this part in the manuscript:

“During metaphase, nucleolar proteins concentrated around the chromosomes along the cell's equator, forming a liquid-like perichromosomal region. At this stage, the equatorial plane of the cell exhibited the highest refractive index. We observed the laser emissions around the equatorial plane.”

[Comment]

2. Figure 6: Data on the nucleolus of various cultured cells should be examined for their validity by comparing them with observations made by other methods.

[Response]

Thanks for the reviewer's suggestion.

To validate our results, we performed nucleolus staining and subsequently conducted a statistical analysis of the nucleoli based on the fluorescence images. These results were then compared with those obtained using the proposed SLEC.

Four cell lines were stained with a commercial nucleolus dye (Nucleolus Bright Red N512, Dojindo), as depicted in Fig. R9.

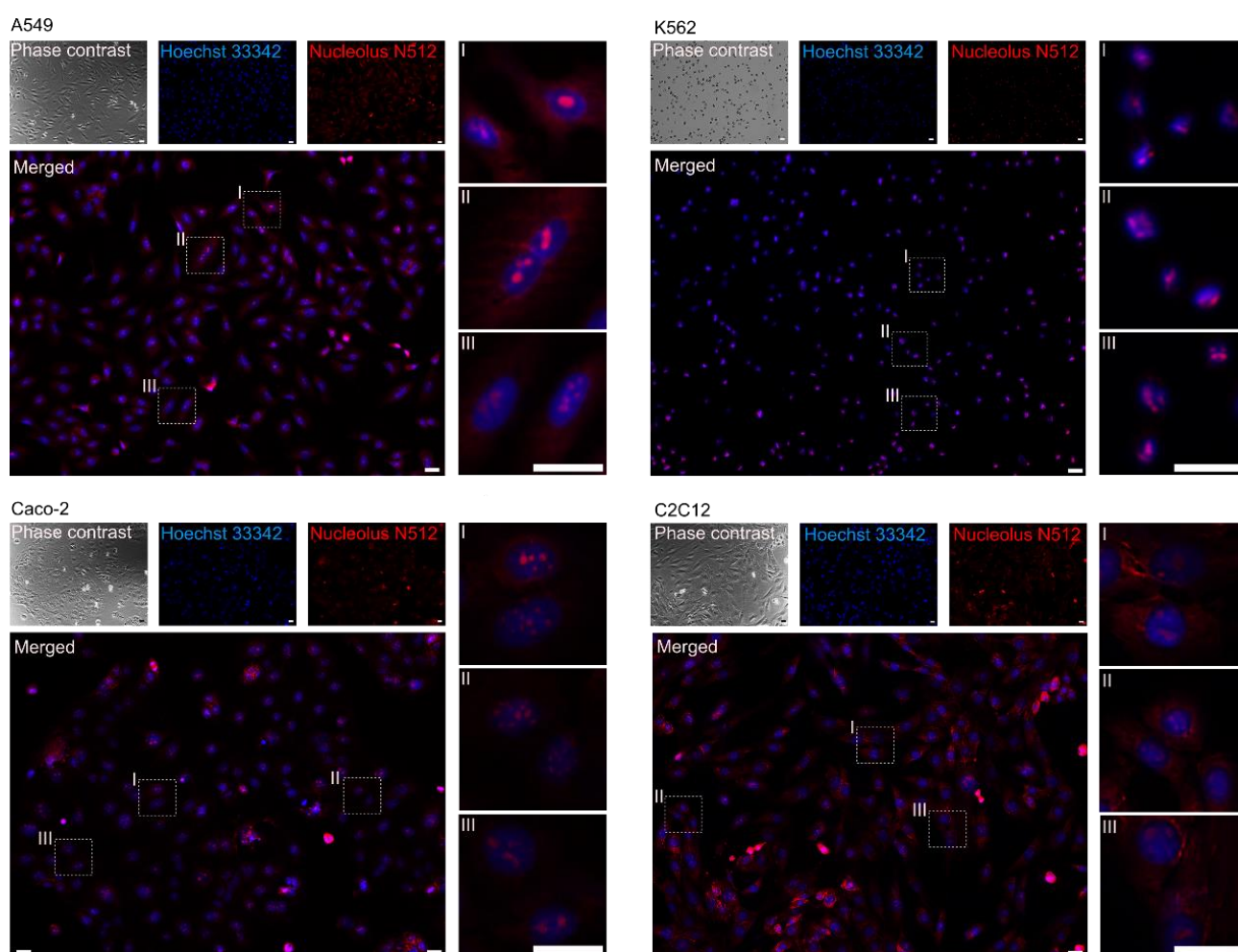


Fig. R9. Images of the nucleolus in A549, K562, Caco-2 and C2C12 cells. The cells were stained with commercial nucleolus dye (Nucleolus Bright Red, N512) and nuclear dye (Hoechst 33342). Scale bar: 20 μm .

With the fluorescence images, we conducted a statistical analysis of nucleoli within different cell types using several parameters: nucleolus number per cell, average nucleolar size, and the

percentage of small nucleoli (Fig. R9).

For A549 and K562 cells, they generally contained between 2 to 5 nucleoli per cell, exhibiting a larger variation in nucleolar size. Approximately half of A549 cells harbored smaller nucleoli ($< 4.5\mu\text{m}^2$) and K562 cells predominantly featured larger nucleoli.

For Caco-2 cells, they had a higher number of nucleoli, ranging from 6 to 9, with a smaller average area compared to A549 and K562 cells. The nucleoli in Caco-2 cells were almost uniformly small.

For C2C12 cells, about half of them contained 2 to 3 nucleoli, and the other half contained 4 to 5 nucleoli. The average nucleolar area in C2C12 cells was small, similar to that observed in Caco-2 cells. Cells with fewer nucleoli tended to have larger nucleoli, whereas those with more nucleoli predominantly had smaller nucleoli.

In general, the results obtained from the image analysis demonstrate a statistical pattern that aligns with those spectral fingerprinting using our proposed SLEC. This consistency underscores the reliability of our lasing method in accurately characterizing nucleolar parameters across different cell types.

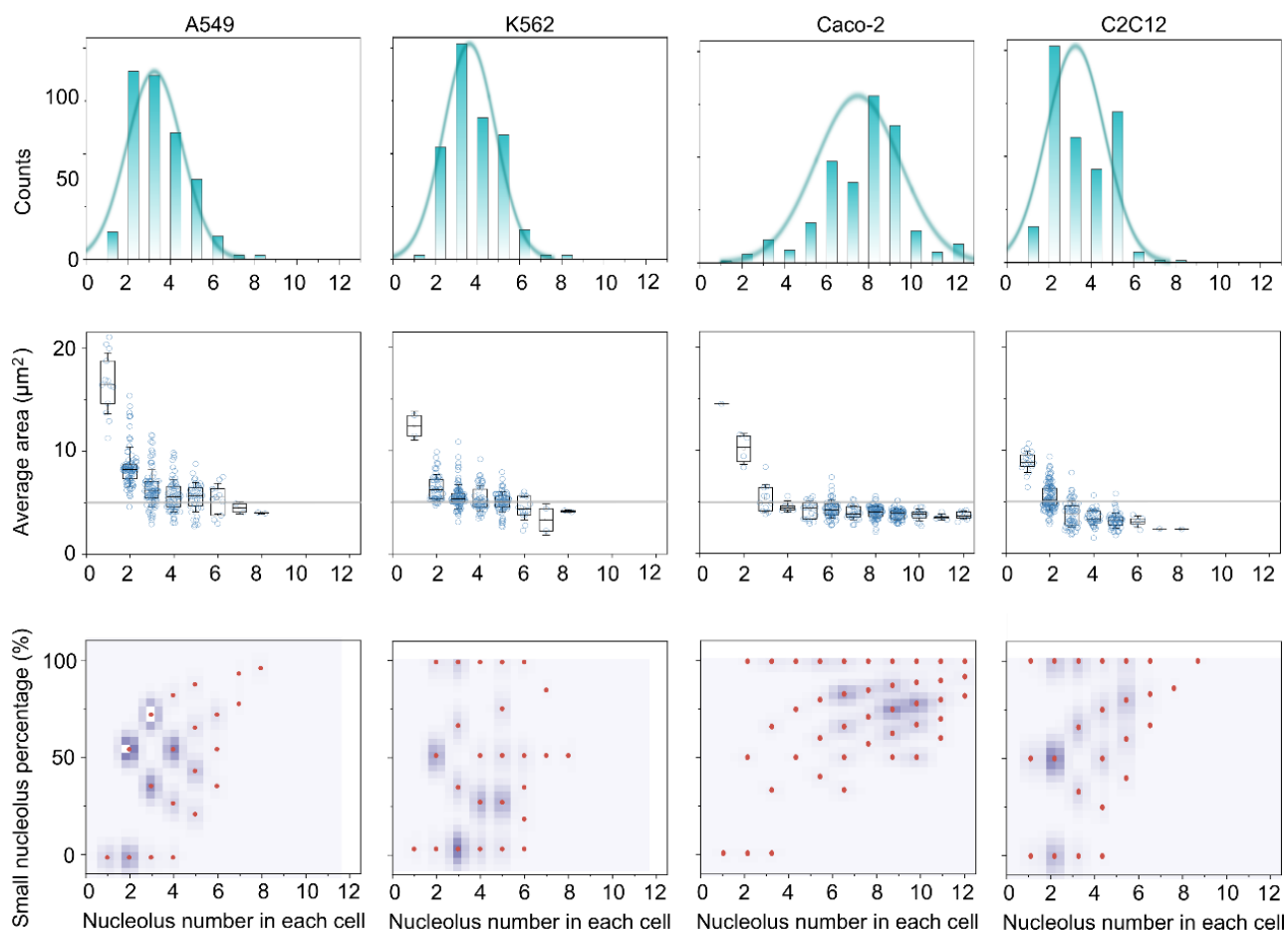


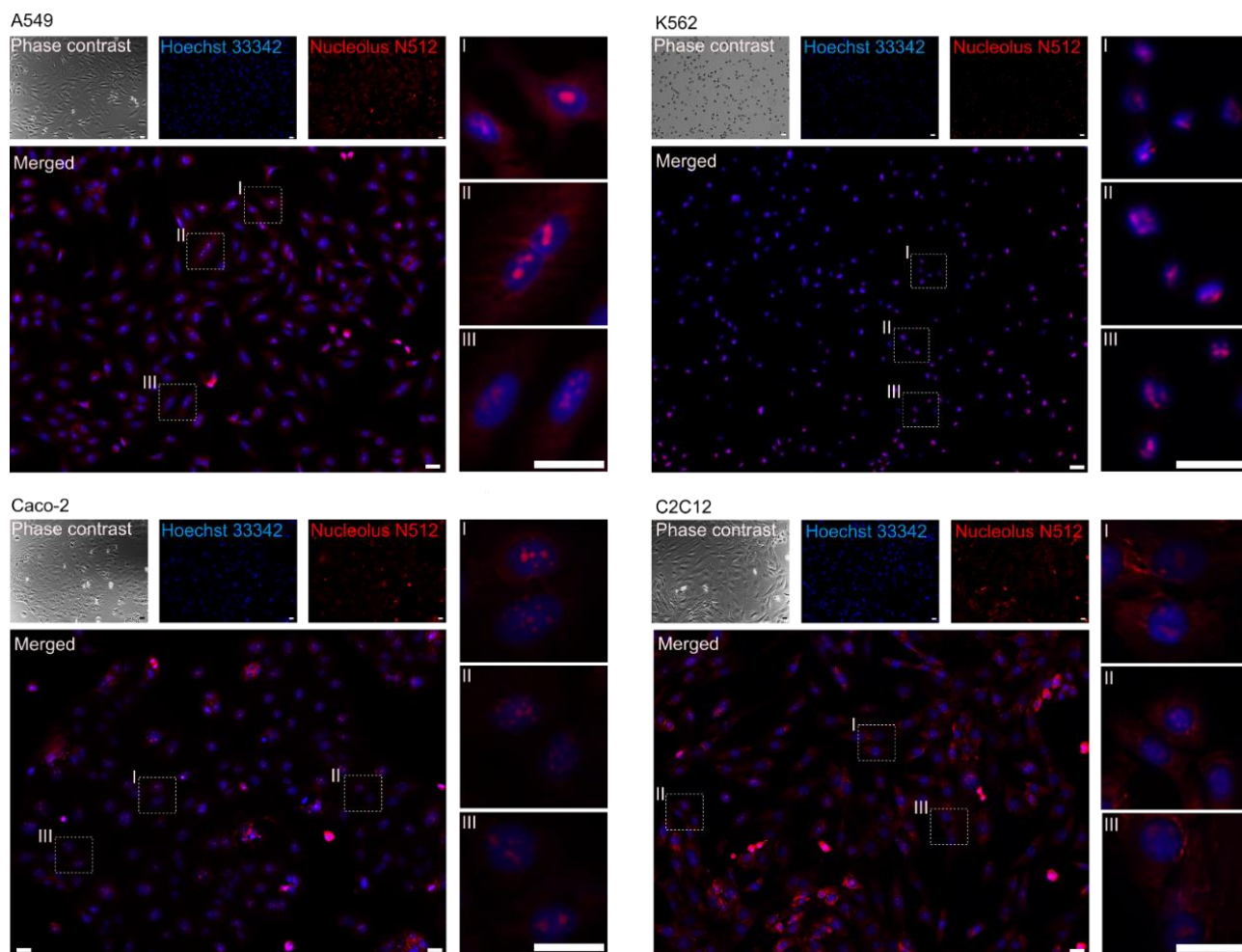
Fig. R10. Nucleolus characteristics from image-based analysis. Nucleolus number, average area of the nucleolus, and percentage of small nucleolus were statistically investigated. (mean $\pm$ SD, N=302 for A549 cells, 301 for K562 cells, 300 for Caco-2 cells, and 300 for C2C12 cells).

[Changes made]

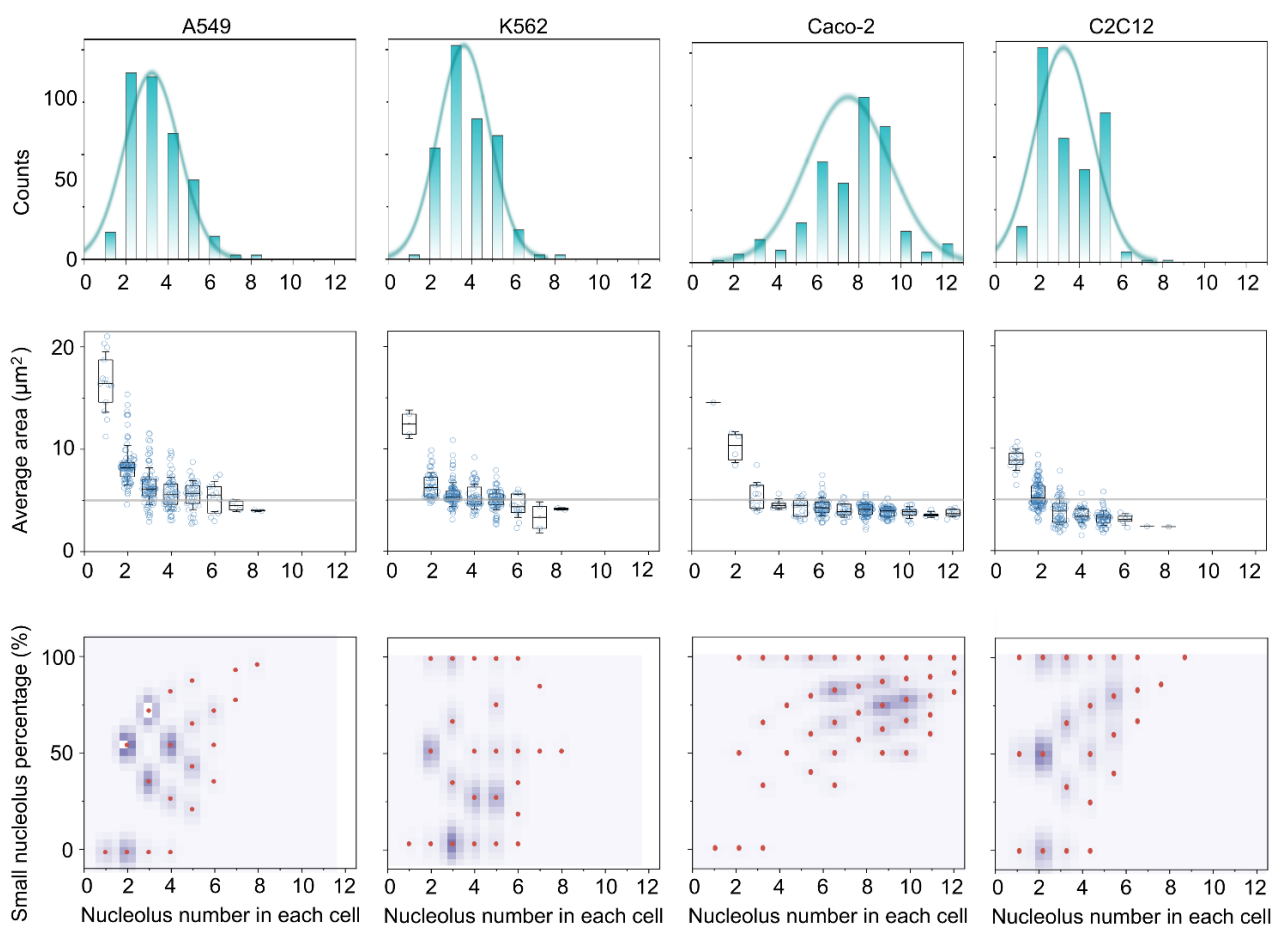
We have added the following sentences in the revised manuscript.

“To valid the result, we stained the nucleoli and conducted a statistical characterization based on the fluorescence images (Figs. S19-20). This revealed the patterns observed in the lasing fingerprinting align well with those derived from the image analysis.”

This validation has been added into the SI as Supplementary Fig. S19 and Supplementary Fig. S20.



Supplementary Fig. S19. Images of the nucleolus in A549, K562, Caco-2 and C2C12 cells. The cells were stained with commercial nucleolus dye (Nucleolus Bright Red, N512) and nuclear dye (Hoechst 33342). Scale bar: 20 μm .



Supplementary Fig. S20. Nucleolus characteristics from fluorescence image-based analysis. Nucleolus number, average area of the nucleolus, and percentage of small nucleolus were statistically investigated. (mean $\pm$ SD, N=302 for A549 cells, 301 for K562 cells, 300 for Caco-2 cells, and 300 for C2C12 cells).

“The results obtained from the image analysis demonstrate a statistical pattern that aligns with those spectral fingerprinting using our proposed SLEC. This consistency underscores the reliability of our lasing method in accurately characterizing nucleolar parameters across different cell types.”

Reviewer #3

[Comment]

The authors describe optical measurements of living cells embedded in a DBR laser cavity. The laser oscillation is facilitated particularly at the nucleolus, where the nucleolus acts as high refractive index lens, enabling label-free imaging with high spatial resolution. The applicability of this method to the suspended cells and flow cytometry is also demonstrated. The achievements are interesting, and the novelty is clear. However, there are several points to be clarified:

[Response]

Thanks for the reviewer's comments. We highly appreciate that the reviewer considers this work is interesting and the novelty is clear. We have addressed all the points raised.

[Comment]

1. Experimental procedures for laser spectroscopic characterization and flow cytometry. The experimental procedures lack some essential information. Particularly, information about the laser scanning should be added. The procedure of the flow cytometry is also unclear. How wide is the channel? How did the authors scan the excitation laser while flowing the cells? How did the authors ensure that all flowing cells were irradiated with the same power? How did the authors irradiate the excitation laser at each area for 5 min while flowing the cells? Add laser images taken outside of the cells and the outside of the nuclei.

[Response]

Thanks for the reviewer's suggestion.

For laser scanning, in order to synchronize with the scanning speed, we replaced the pump laser with a high repetition rate pulsed laser (Microlaser SB1-473-3-5, 5 kHz, 2 ns pulse width). The pump laser was focused to a spot with a diameter of approximately 34 μm . A scanning step of 5 μm was configured using the controller in the Nikon Ni-E upright confocal microscopy. Images were captured using a fast camera (Photron, FASTCAM Mini UX 50) with an exposure time of 2 ms.

In the flow cytometry setup, SU-8 was spin-coated on the bottom mirror with a thickness of $\sim 35 \mu\text{m}$. The width of the flow channel at the pump area was designed to be 35 μm to allow single-cell passage, while at other locations, the channel width was $\sim 200 \mu\text{m}$. To prevent cell blockage, pillar filters with a distance of 25 μm were incorporated at the inlet.

The pulsed lasers in this study had repetition rates of 50Hz and 5kHz. The pump spot diameter was 72 μm . It was set to continuously pump the same area of the channel with consistent energy for 5 mins. This setup ensured that as cells flowed through the channel, they could be immediately exposed to the laser pulses. With a constant flow speed, the cells thus could receive the same power. In practice, merely 1-3 pulses were sufficient to fully excite each cell. Thus, even when cells passed through the channel at high speeds, they were adequately pumped. This process necessitated careful coordination between the cell flow speed and the laser repetition rate.

[Changes made]

Following the reviewer's suggestion, we have revised the following information in the manuscript.

“Cell scanning: In order to synchronize with the scanning speed, we replaced the pump laser with a high repetition rate pulsed laser (Microlaser SB1-473-3-5, 5 kHz). The pump laser was focused to a spot with a diameter of 34 μm . A scanning step of 5 μm was configured using the controller in the Nikon Ni-E upright confocal microscopy. Images were captured using a fast camera (Photron, FASTCAM Mini UX 50) with an exposure time of 2 ms.”

“Subsequently, a layer of SU-8 (2000.5, KAYAKU) with a thickness of $\sim 0.5 \mu\text{m}$ was spin-coated to enhance adhesion. Then a 35 μm -thick layer of SU-8 (2010, KAYAKU) was coated for the microwells and flow cytometry channels. The microstructures were projected on the SU-8 layer by a UV lithographer (UV Litho-ACA, TuoTuo Technology Pte. Ltd.). The microwell array was designed with a diameter of $\sim 40 \mu\text{m}$. The width of the flow channel at the pump area was designed as 35 μm to allow single-cell passage, while at other locations, the channel width was $\sim 200 \mu\text{m}$. To prevent cell blockage, pillar filters with a distance of 25 μm were incorporated at the inlet.”

Laser images taken outside of the cells and the outside of the nuclei have been added in Fig. 3c as following:

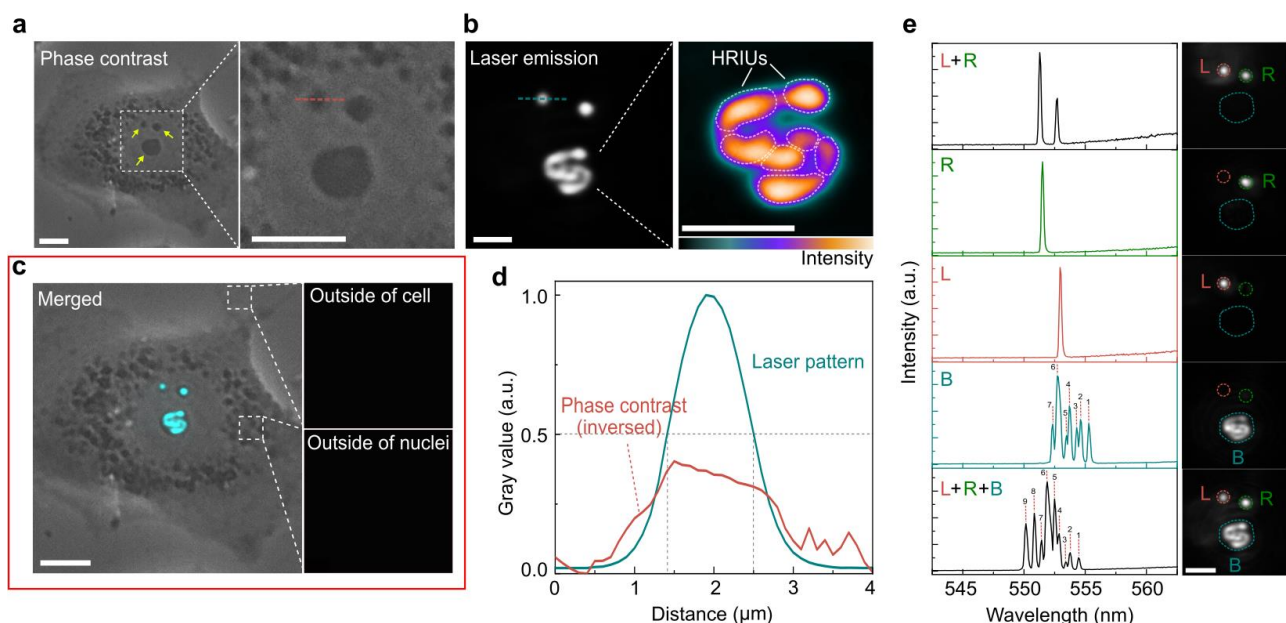


Fig. 3 | Characterization of the laser emission for nucleolus in spreading A549 cells. **a**, Phase contrast image depicting a live A549 cell, with a zoomed-in section highlighting the nucleoli. Scale bar: 10 μm . **b**, Laser emission image within the nucleoli. The separated laser spots were designated as HRIUs within the nucleoli. Scale bar: 5 μm . **c**, Merged image of the phase contrast image and laser emission image. Scale bar: 10 μm . **No laser emission was observed when pumping the areas outside of the cell and outside of the nuclei (inset images).**

[Comment]

2. Discussions about Fig. 4. Observe the time-course change in the peak wavelength to validate their claim written in page 9 (the laser's high sensitivity to refractive index changes). In addition, repeat the experiments for Fig. 4c and e for several times and give error bars for supporting the quantitative discussion.

[Response]

Thanks for the reviewer's suggestion. We added the time-course change in laser peaks of the live cells (Fig. R11). It could be seen that for this live cell, the laser spectrum changed significantly with time. The laser peak number ranged from 4 to 8. In live cells, the flow-like change of molecules within nucleolus induced the spatial change of the refractive index, which resulted in the significant change in spectrum.

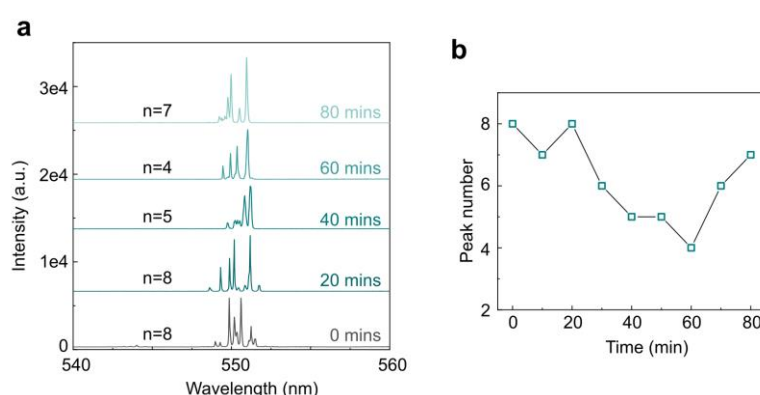


Fig. R11. Laser spectrum change for the live cell. (a) Laser spectrum at 0, 20, 40, 60 and 80 mins, respectively. n represents the number of laser peaks. (b) Laser peak number change with time.

Followed the reviewer's suggestion, we have repeated the experiments and added the error bars in Fig. 4e.

Because the Fig. 4c illustrates the changes in the cell depicted in Figure 4a, thus statistical analysis is not required. However, to achieve the quantitative discussion, we analyzed various cells and statistically recorded the maximum changes in gray value over a duration of 80 mins. This revealed that the maximum change in gray value in live cells was ~ 12.8 times greater than that observed in fixed cells (Fig. R12).

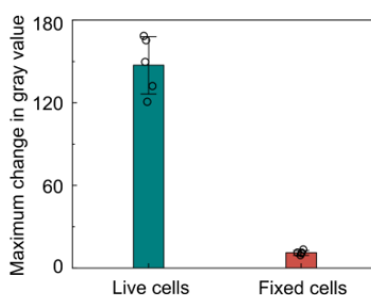
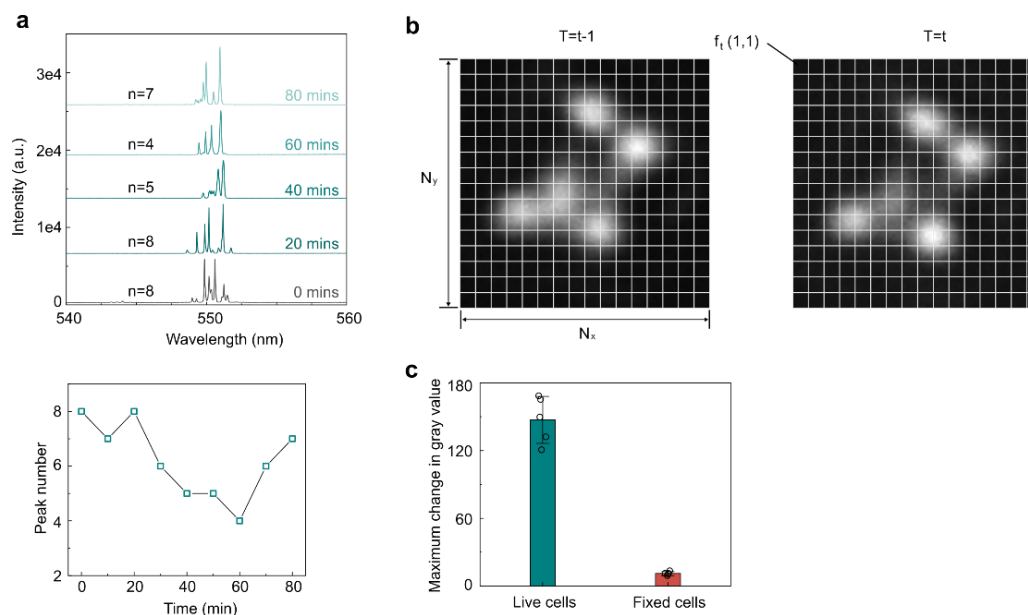


Fig. R12. Comparison of the maximum change in gray value between live cells and fixed cells (mean $\pm$ SD, N=5).

[Changes made]

We have added these figures into the Supplementary Fig. S12, as seen below:



Supplementary Fig. S12. Characterization of laser emission change. (a) Laser spectrum change of the live cell in fig. 4a. (b) Calculation of the alterations in the laser emission image. (c) Comparison of the maximum change in gray value between live cells and fixed cells (mean $\pm$ SD, N=5).

The following sentences have been added into the revised manuscript.

“The laser spectrum also showed obvious fluctuation (Fig. S12a).”

“The gray value fluctuation in live cells was $\sim$ 12.8-fold larger than that in the fixed cells (Fig. 4c, S12c).”

[Comment]

3. Cytotoxicity. The description on the cytotoxicity should be written fairly. The laser threshold reported herein is rather high among organic lasers. Thus, the reviewer wonders why such intense laser did not harm the living cells and find that the repetition rate of the excitation laser was so slow (50 Hz) in comparison to the typical pulsed lasers (1 kHz). The authors should make this point clear in the manuscript and fairly evaluate the cytotoxicity of their method in comparison to the other optical techniques.

[Response]

Thanks for the reviewer's comments. We have included a summary of recent studies on microlasers in biological applications in Table R2. The energy intensity ranged from 0.3 to 183 $\mu\text{J mm}^{-2}$. The laser threshold of this proposed device is 21-68 $\mu\text{J mm}^{-2}$, which is close to the energy intensity of these microlasers. It is reported that when the pump intensity increased beyond 1 $\mu\text{J/pulse}$ ($\sim 318.3 \mu\text{J mm}^{-2}$), the cells were physically damaged (Gather, M., et al., Nature Photon., 2011, 5, 406-410; Matjaž Humar, et al., Opt. Express., 2015, 23, 21). In our experiment of the cell toxicity, no cell damage was observed after 2-mins exposure under 68.3 $\mu\text{J mm}^{-2}$ (Supplementary Fig. S10). Therefore, the intensity of the pump laser in this study should be within a safety range for cell applications.

Table R2 Energy intensity and repetition rate of the microlasers in biological applications

Configuration	Energy intensity used	Repetition rate	Laser type	Paper
Single cell sandwiched between two mirrors (GFP)	14 nJ = 44.6 $\mu\text{J mm}^{-2}$	10 Hz	F-P	Malte C. Gather, et al., Nat. Photon, 2011, 5, 406-410.,
Cell sandwiched between two mirrors (dye-labeled)	50 - 100 $\mu\text{J mm}^{-2}$	10 Hz	F-P	Matjaž Humar, et al., Opt. Express., 2015, 23, 21.
Cell sandwiched between two mirrors	40 $\mu\text{J mm}^{-2}$	50 Hz, 5 kHz	F-P	Chaoyang Gong, et al., Laser Photonics Rev., 2022, 2100734
Cell sandwiched between two mirrors (dye-labeled)	68 $\mu\text{J mm}^{-2}$	50 Hz	F-P	Zhen Qiao, et al., Adv. Sci., 2022, 9, 2103550
Dye droplets in cells	3 - 30 nJ (9.6 - 95.6 $\mu\text{J mm}^{-2}$)	10 Hz	WGM	Matjaž Humar, et al., Nat. Photon, 2015, 9, 572-576.
Semiconductor disks in cells	12.7 $\mu\text{J mm}^{-2}$	N.A.	WGM	Nicola Martino, et al., Nat. Photon, 2019, 13, 720-727.
PS sphere in cells	0.5 - 7 nJ (0.3 - 4.2 $\mu\text{J mm}^{-2}$)	10 Hz	WGM	Marcel Schubert, et al., Nano Lett., 2015, 15, 5647-5652.
Droplet spheres with bacterias inside	183 - 554 $\mu\text{J mm}^{-2}$	80 MHz	WGM	Alexandr Jonas, et al., Lab. Chip, 2014, 14, 3093.
Cell sandwiched between two mirrors (non-labeled)	68.3 $\mu\text{J mm}^{-2}$	50 Hz, 5 kHz	F-P	This work

Note: The diameter of laser beam was considered as 20 μm during unit conversion.

From Table R2, the repetition rate ranged from 10 Hz to 80 MHz in these studies. We have conducted further tests on our device using a pump laser with a 5 kHz repetition rate and confirmed that the laser signal can be achieved. Thus, the proposed device is capable of functioning effectively across a wide range of repetition rates. We further tested the device under a pump laser with 5 kHz

repetition rate, the laser signal can be achieved. Thus, the proposed device can work at both low and high repetition rate.

[Changes made]

Followed the reviewer's suggestion, we have added the relevant data to Supplementary Table S1 and included a discussion on cytotoxicity in the manuscript.

“This pump energy is comparable to other reported laser in biological applications and no cell damage was observed (Fig. S10, Table S1).”

Table S1 Energy intensity and repetition rate of microlasers in biological applications

Configuration	Energy intensity used	Repetition rate	Laser type	Paper
Single cell sandwiched between two mirrors (GFP)	14 nJ = 44.6 $\mu\text{J mm}^{-2}$	10 Hz	F-P	Malte C. Gather, et al., Nat. Photon, 2011, 5, 406-410.,
Cell sandwiched between two mirrors (dye-labeled)	50 - 100 $\mu\text{J mm}^{-2}$	10 Hz	F-P	Matjaž Humar, et al., Opt. Express., 2015, 23, 21.
Cell sandwiched between two mirrors	40 $\mu\text{J mm}^{-2}$	50 Hz, 5 kHz	F-P	Chaoyang Gong, et al., Laser Photonics Rev., 2022, 2100734
Cell sandwiched between two mirrors (dye-labeled)	68 $\mu\text{J mm}^{-2}$	50 Hz	F-P	Zhen Qiao, et al., Adv. Sci., 2022, 9, 2103550
Dye droplets in cells	3 - 30 nJ (9.6 - 95.6 $\mu\text{J mm}^{-2}$)	10 Hz	WGM	Matjaž Humar, et al., Nat. Photon, 2015, 9, 572-576.
Semiconductor disks in cells	12.7 $\mu\text{J mm}^{-2}$	N.A.	WGM	Nicola Martino, et al., Nat. Photon, 2019, 13, 720-727.
PS sphere in cells	0.5 - 7 nJ (0.3 - 4.2 $\mu\text{J mm}^{-2}$)	10 Hz	WGM	Marcel Schubert, et al., Nano Lett., 2015, 15, 5647-5652.
Droplet spheres with bacterias inside	183 - 554 $\mu\text{J mm}^{-2}$	80 MHz	WGM	Alexandr Jonas, et al., Lab. Chip, 2014, 14, 3093.
Cell sandwiched between two mirrors (non-labeled)	68.3 $\mu\text{J mm}^{-2}$	50 Hz, 5 kHz	F-P	This work

Note: The diameter of laser beam was considered as 20 μm during unit conversion.

“The pump laser demonstrated cell damage when the intensity exceeded approximately 1 μJ , equivalent to about 318.3 $\mu\text{J mm}^{-2}$, given a laser beam diameter of 20 μm . Thus, the pump energy used in this study is considered safe for the cells.”

[Comment]

4. Comparison with the Reference 24 (M. C. Gather et al., Nat. Photon. 2011) reports analogous experimental results, where living cell was sandwiched inside a laser cavity. Nonetheless, the laser emission is homogeneous over the whole cell rather than specific to nucleolus. Provide a reasonable explanation for this difference.

[Response]

Thanks for the reviewer's insightful comments. This phenomenon observed in our study is indeed compelling. Compared our result with the M.C. Gather's research, we observed distinct and pronounced laser emission in the nucleolar area (Fig. R13). Notably, even under high pump intensity that trigger laser emissions across the entire cell, the nucleolar region consistently exhibited the most intense emissions.

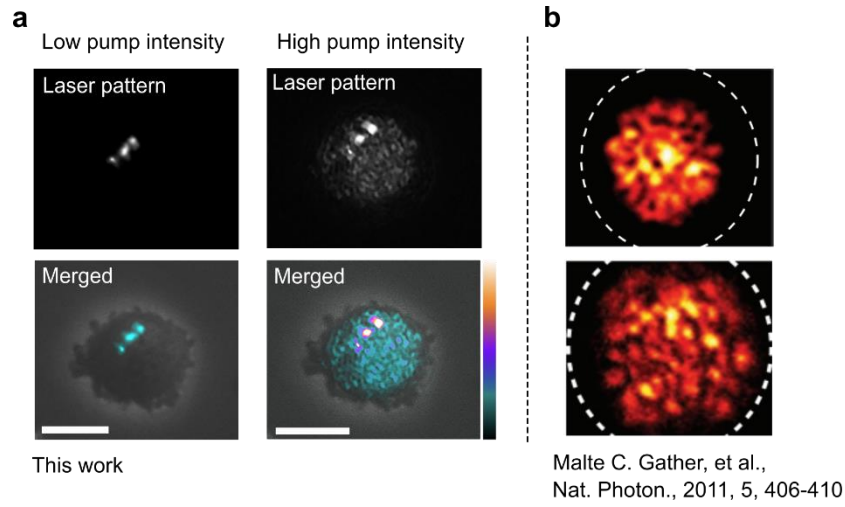


Fig. R13 Comparison of the laser emission images. (a) Laser emission from the cells in our work. At low pump intensity, only nucleolar area generated laser emission. At high pump intensity (e.g. $143.5 \mu\text{J mm}^{-2}$), the entire cell generated laser emission. But the nucleolar area showed the highest emission intensity. Scale bar: 10 μm . (b) Laser emission in M.C. Gather's work.

We attribute these observations to differences in laser threshold, governed by the properties of the gain medium (volume, distribution, etc.). Below is the theoretical analysis:

The threshold pump power for lasing can be written as Eq. (R1).

$$P_{th} = \frac{h\nu_p}{2\Delta d\sigma_l\tau_f\eta_c[1 - \exp(-\alpha\Delta d)]} \cdot \frac{L}{\iiint S(r, \varphi, z)H(r, \varphi, z)dV} \quad (\text{R1})$$

where h is the Plank constant, ν_p is the frequency of the pump light, σ_l is the laser transition cross section, τ_f is the fluorescence lifetime, η_c is the ratio of the pump power coupled in the cavity to the incident pump power, Δd is the length of the gain medium, α is the absorption coefficient for the pump light, L is the round-trip cavity loss, and $S(r, \varphi, z)$ and $H(r, \varphi, z)$ are the normalized three-dimensional intensity distributions of pump light and laser light, respectively, under the cylindrical coordinate system (r, φ, z) . Consider that the length of the gain medium is very small, the following approximation is satisfied:

$$1 - \exp(-\alpha\Delta d) \approx \alpha\Delta d \quad (\text{R2})$$

In consideration of the diminutive length of the cavity, the diffractive effects of the laser light within the cavity can be neglected. Thus, the subsequent approximation can be obtained:

$$\iiint S(r, \varphi, z)H(r, \varphi, z)dV \approx \frac{1}{\Delta d} \iint s(r, \varphi)h(r, \varphi)rdrd\varphi \quad (R3)$$

where $s(r, \varphi)$ and $h(r, \varphi)$ are the normalized two-dimensional intensity distributions of pump light and laser light, respectively, under the polar coordinate system (r, φ) . To further simplify Eq. (1), we define the effective mode area of a laser mode as Eq. (R4).

$$A = \frac{1}{\iint s(r, \varphi)h(r, \varphi)rdrd\varphi} \quad (R4)$$

Substituting Eqs. (R2)-(R4) into Eq. (R1), we have:

$$P_{th} = \frac{1}{\Delta d} \cdot \frac{h\nu_p LA}{2\alpha\sigma_1\tau_f\eta_c} \quad (R5)$$

Equation (R5) reveals that the threshold pump power is inversely proportional to the length of the gain medium. In the case of our work, the length of the gain medium (PS layer) is substantially reduced. Consequently, the threshold pump power for a specific laser mode is significantly elevated. Additionally, the disparity between the thresholds of lower-order and higher-order laser modes is augmented. The Fig. R14-b elucidates the impact of the gain medium's length (Δd) on the pump threshold. At a given pump power, laser modes with larger effective mode areas can be obtained under a larger Δd . This means that higher-order laser modes can be excited. Conversely, only lower-order laser modes with smaller effective mode areas can be excited under a smaller Δd . Therefore, in a labelled cell laser, higher-order laser modes can be excited, resulting in an expansive laser pattern that covers the entire cellular structure.

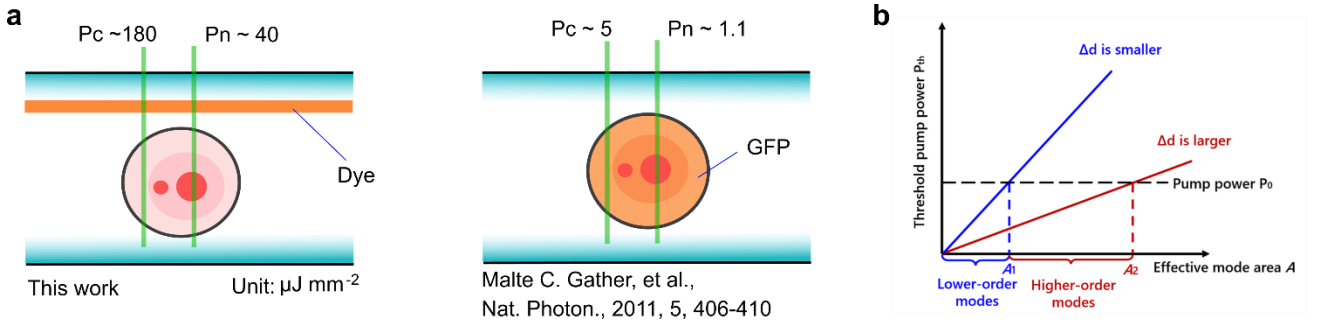


Fig. R14. Comparison of our work (label-free cell laser) and M.C. Gather's work (labeled cell laser) in threshold. (a) Configuration of the two works. In our study, due to the increased threshold, the threshold difference between nucleolar area and cytoplasm can reach $\sim 140 \mu\text{J mm}^{-2}$. In M.C. Gather's research, the gain medium was considered to saturate the entire cavity, resulting in a minimal threshold difference of $\sim 3.9 \mu\text{J mm}^{-2}$. (b) Relationship of threshold, effective mode area, and gain medium distribution.

In our experiments, the gain medium comprised a PS laser, spin-coated onto the top mirror with a merely 1-2 μm thickness, accounting for ~ 11 -22% of the cavity volume. In this case, the laser threshold in the cytoplasm (Pc) reached $\sim 180 \mu\text{J mm}^{-2}$, and the nucleolar area (Pn) exhibited an average threshold of $\sim 40 \mu\text{J mm}^{-2}$, representing a decrease of $\sim 77\%$ attributable to the lens effect. The threshold difference reached $\sim 140 \mu\text{J mm}^{-2}$, facilitating laser emission at the nucleolar area with

only lower-order modes.

In contrast, M.C. Gather utilized green fluorescent proteins (GFP) as the gain medium, which were distributed uniformly across the cytoplasm and nucleus, completely filling the whole cavity. In such a setup, the threshold in the cytoplasm (P_c) was $\sim 5 \mu\text{J mm}^{-2}$. Assuming a similar lens effect in the nucleolus, the threshold in nucleolar area would be expected to $\sim 1.1 \mu\text{J mm}^{-2}$. The threshold difference was only $3.9 \mu\text{J mm}^{-2}$, resulting in both lower and higher modes over the whole cell.

Therefore, our design enhances the threshold difference by ~ 36 -fold, markedly improving the discernibility of heterogeneous lasing emissions within cells. Moreover, this configuration ensures predominant laser emissions from the lower-order modes, thereby facilitating spectrum-based analysis.

[Changes made]

To better illustrate the difference of our design compared to previous studies, we have added the following theoretical analysis as the Supplementary Note 1.

In our study, we observed that the lasers selectively emitted from the nucleolar area, with most emissions displaying single-order mode characteristics. We attribute these observations to disparities in laser threshold levels.

The threshold pump power for lasing can be written as Eq. (N1).

$$P_{th} = \frac{h\nu_p}{2\Delta d\sigma_i\tau_f\eta_c[1-\exp(-\alpha\Delta d)]} \cdot \frac{L}{\iiint S(r, \varphi, z)H(r, \varphi, z)dV} \quad (\text{N1})$$

where h is the Plank constant, ν_p is the frequency of the pump light, σ_i is the laser transition cross section, τ_f is the fluorescence lifetime, η_c is the ratio of the pump power coupled in the cavity to the incident pump power, Δd is the length of the gain medium, α is the absorption coefficient for the pump light, L is the round-trip cavity loss, and $S(r, \varphi, z)$ and $H(r, \varphi, z)$ are the normalized three-dimensional intensity distributions of pump light and laser light, respectively, under the cylindrical coordinate system (r, φ, z) . Consider that the length of the gain medium is very small, the following approximation is satisfied:

$$1 - \exp(-\alpha\Delta d) \approx \alpha\Delta d \quad (\text{N2})$$

In consideration of the diminutive length of the cavity, the diffractive effects of the laser light within the cavity can be neglected. Thus, the subsequent approximation can be obtained:

$$\iiint S(r, \varphi, z)H(r, \varphi, z)dV \approx \frac{1}{\Delta d} \iint s(r, \varphi)h(r, \varphi)rdrd\varphi \quad (\text{N3})$$

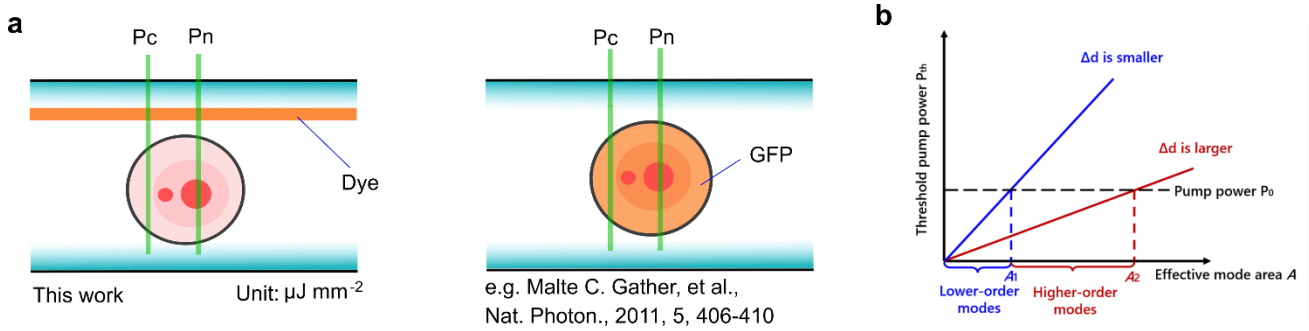
where $s(r, \varphi)$ and $h(r, \varphi)$ are the normalized two-dimensional intensity distributions of pump light and laser light, respectively, under the polar coordinate system (r, φ) . To further simplify Eq. (1), we define the effective mode area of a laser mode as Eq. (R4).

$$A = \frac{1}{\iint s(r, \varphi)h(r, \varphi)rdrd\varphi} \quad (\text{N4})$$

Substituting Eqs. (N2)-(N4) into Eq. (N1), we have:

$$P_{th} = \frac{1}{\Delta d} \cdot \frac{h\nu_p LA}{2\alpha\sigma_l\tau_f\eta_c} \quad (\text{N5})$$

Equation (N5) reveals that the threshold pump power is inversely proportional to the length of the gain medium. In the case of our work, the length of the gain medium (PS layer) is substantially reduced. Consequently, the threshold pump power for a specific laser mode is significantly elevated. Additionally, the disparity between the thresholds of lower-order and higher-order laser modes is augmented. The Fig. N1-b elucidates the impact of the gain medium's length (Δd) on the pump threshold. At a given pump power, laser modes with larger effective mode areas can be obtained under a larger Δd . This means that higher-order laser modes can be excited. Conversely, only lower-order laser modes with smaller effective mode areas can be excited under a smaller Δd . Therefore, in a labeled cell laser, higher-order laser modes can be excited, resulting in an expansive laser pattern that covers the entire cellular structure.



Supplementary Note Fig. N1. Comparison of our work (label-free cell laser) and the previous work (labeled cell laser) in threshold. (a) Configuration of the devices. In this work, due to the increased threshold, the threshold difference between nucleolar area and cytoplasm significantly increased. In previous research, the cell was labeled and the gain medium saturated the entire cavity. Therefore, the threshold was relatively low, resulting in a minimal threshold difference. (b) Relationship of threshold, effective mode area, and gain medium distribution.

In our work, the laser threshold in the cytoplasm (P_c) reached $\sim 180 \mu\text{J mm}^{-2}$, and the nucleolar area (P_n) exhibited an average threshold of $\sim 40 \mu\text{J mm}^{-2}$. The threshold difference reached $\sim 140 \mu\text{J mm}^{-2}$, facilitating laser emission at the nucleolar area with only lower-order modes. In the case of labeled cells, the threshold in the cytoplasm (P_c) was $\sim 5 \mu\text{J mm}^{-2}$. Assuming a similar lens effect in the nucleolus, the threshold in nucleolar area would be expected to $\sim 1.1 \mu\text{J mm}^{-2}$. The threshold difference was only $3.9 \mu\text{J mm}^{-2}$, resulting in both lower and higher modes over the whole cell.

Therefore, the design of separating gain medium in our work enhances the threshold difference by ~ 36 -fold, markedly improving the discernibility of heterogeneous lasing emissions within cells. Moreover, this configuration ensures predominant laser emissions from the lower-order mode, thereby facilitating spectrum-based analysis.

[Comment]

5. Novelty of the method. In my opinion, the novelty of the methodology reported in the manuscript is not very high because the method is mostly identical to those written in the reference 24 except the location of the gain. The authors claim that the method is label-free, but it is realized at the expense of the target-specificity. It is in clear contrast to the conventional label-free techniques (Raman microscope, for instance) that have been developed so that they can identify molecules and proteins without label.

[Response]

We respectfully disagree with the reviewer regarding the novelty of our methodology. The technique introduced in our study encapsulates significant innovations, detailed as follows:

1. **Label-free analysis through laser emission signals:** Our method achieves label-free nucleolar analysis by utilizing laser emission signals. This is a breakthrough in laser emission technique. Distinct from conventional techniques that necessitate the integration of the gain medium within the samples, our approach strategically separates the gain medium from the samples and utilizes the refractive index difference. This fundamental distinction not only simplifies the sample processing but also significantly extends the applicability to clinical samples, including patient-derived specimens. This represents a methodological advancement over approach in reference 24, which relies on green fluorescent proteins (GFP) or dye as the gain medium and may pose considerable challenges for clinical applications.
2. **New lasing mechanism to enhance the threshold difference within intracellular structures and thereby enabling the strong linkage between laser emission and specific intracellular structures:** As discussed in Question 4, our design notably increases the threshold difference between the cytoplasm and nucleolus by ~36-fold, allowing laser emissions to be specifically regulated by the nucleolar area. In addition, it avoids the laser emission with high-order modes. Consequently, the laser emission attributes can effectively reflect the features of nucleoli such as number, size, and liquid-like flowing activities. This is a significant advancement over reference 24, where laser emission is homogeneous across the cell, making it difficult to correlate specific intracellular information with the laser emissions.
3. **Flow cytometry for nucleolus analysis via lasing emission fingerprinting:** Previous studies have generally been static in their investigation of cells. The first single-cell biological laser (Gather, M., et al., Nature Photon., 2011, 5, 406-410), sparked anticipation for its potential application in flow cytometry. However, the conventional laser emissions usually generate low-order modes along with high-order modes, which mask the efficient information. The separation design of gain can effectively eliminate the high-order modes and remain the low order modes. Thus, the laser image or spectrum could reflect the efficient information.

Therefore, our approach achieves flow cytometry for nucleolar analysis, enabling throughput up to 2,500 events per second (eps). Moreover, due to the specific regulation of laser emissions by the nucleolus, the developed flow cytometry technique can precisely display statistical information related to nucleoli, thus providing insights into nucleolus-associated diseases.

In terms of the expense of the target-specificity, we believe this represents a significant improvement in the field. As we mentioned above, although laser emissions from cells can provide distinct and high-contrast signals, linking these emissions to specific organelles or structures has been a longstanding challenge, limiting the application of laser technologies in more precise studies and disease-specific investigations. Our study uniquely focuses laser emissions on the nucleolus, enabling detailed exploration of nucleolar morphology and biophysical activities. As the nucleolus gains increasing recognition in disease detection and liquid-liquid phase separation (Tiku V., et al., *Nature Communications*, 2017, 18, 651; Zlotorynski, et al., *Nature Reviews Molecular Cell Biology*, 2018, 28, 662-672; Mitrea D.M. et al., *Nature Communications*, 2018, 9, 842; Lafontaine D.L.J., *Nature Reviews Molecular Cell Biology*, 2021, 22, 165-182), our work presents a valuable tool for studying nucleolar diseases and models, especially noteworthy as it is achieved through a label-free approach applicable to both live and dead cells - capabilities not offered by previous studies.

When compared to conventional label-free techniques such as Raman microscopy, our method exhibits significant advantages:

1. **Ultrahigh signal-to-noise ratio (SNR):** Our approach capitalizes on the inherent light amplification properties of laser emissions, resulting in an ultrahigh SNR ranging from ~ 400 to 2000, which can increase further with higher pump intensities. In contrast, the Raman effect is inherently inefficient, typically producing weak signals with an SNR between 3 and 150, heavily dependent on integration time (Sinead J. Barton et al., *Biophotonics: Photonic Solutions for Better Health Care VI*, 2018, 106854F; Xian-guang Fan et al., *J Raman Spectrosc.* 2021, 52:890-900). The SNR is calculated by dividing the difference between the peak signal and the background signal by the root mean square (RMS) value of the noise on the background signal.

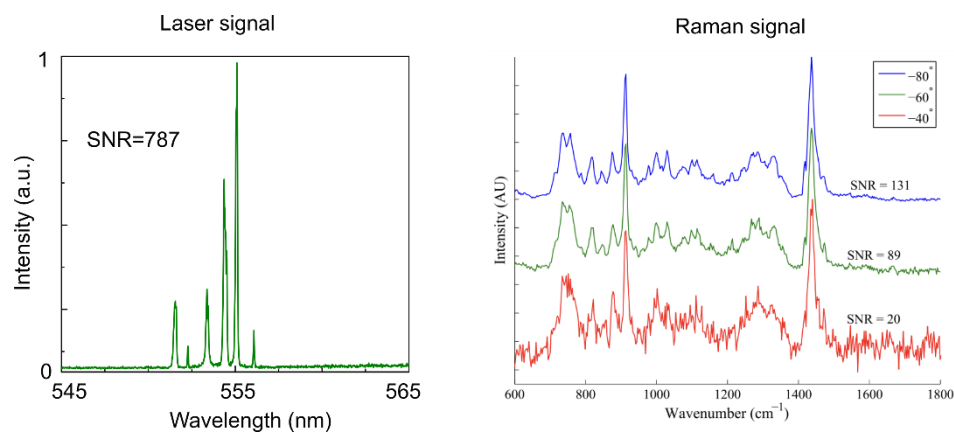


Fig. R15 Comparison of laser signal in our method and the Raman signal. (a) A typical laser spectrum with SNR of ~ 787 . (b) The Raman signal cited in reference ‘Sinead J. Barton, et al., *Biophotonics: Photonic Solutions for Better Health Care VI*, 2018, 106854F’. The SNRs of the Raman signal are 20, 89 and 131, respectively.

2. **Ultrafast acquisition speed of laser emission:** Our system achieves a rapid acquisition time, capable of collecting data from a single cell within 0.5 ms. This rapid acquisition enables the real-time observation of dynamic activities within the nucleolus of live cells. Due to high SNR of the laser signal, there is no need for prolonged integration time, allowing immediate collection of

the laser signal post-pumping. The speed of laser signal collection is contingent upon the repetition rate of the pulsed laser and the acquisition speed of the spectrometer, which are both on the order of kHz.

In contrast, Raman signals, being inherently weak, generally require longer acquisition times to achieve a good SNR. This prolonged data collection is usually problematic for live cell imaging, as cells are dynamic and may move or alter state during the data collection period. For example, systems like Raman-activated microbial cell sorting analyze only 3.3-8.3 cells per minute (Kang Soo Lee et al., Nature Microbiology, 2019, 4:1035-1048). Even with recent advancements in Raman image-based cell analysis that achieve around 100 eps (Nao Nitta et al., Nature Communications, 2020, 11:3452), it remains challenging to perform fast analysis with strong signal quality in this field.

[Changes made]

In order to highlight the innovation, the abstract has been revised.

Nucleolus, a recognized biomolecular condensate, serves as the hub for ribosome biogenesis within cell nucleus. Its quantity and morphology are discernible indicators of cellular functional states. However, precise identification and quantification of nucleolus remains challenging without specific labeling, particularly for suspended cells, [tissue-level analysis](#) and high-throughput applications. Here we introduce a single-cell laser emitting cytometry (SLEC) for label-free nucleolus differentiation through light-matter interactions within a Fabry-Perot photonic resonator. [The separated gain medium enhances the threshold difference by 36-fold between nucleolus and its surroundings, enabling selective laser emissions at nucleolar area while maintaining lower-order mode.](#) The laser emission image provides insights into structural inhomogeneity, temporal fluid-like dynamics, [and pathological application.](#) Lasing spectral fingerprint depicts the quantity and size of nucleoli within a single cell, showcasing the label-free flow cytometry for nucleolus. This approach holds promise for nucleolus-guided cell screening and drug evaluation, advancing the study of diseases such as cancer and neurodegenerative disorders.

The introduction has been revised by adding the following sentences.

[‘Probe-labeled techniques offer a high-level specificity for nucleolus targeting, which requires either particular labeling chemistry \(dye, specific antibodies, etc.\) or expression of fluorescence-tagged proteins for direct detection²³. However, the artificial probes make it challenging to study the native cellular interactions and processes. Furthermore, fluorescence-based techniques often contend with strong background noise.’](#)

[‘However, most laser emissions still fall short in furnishing essential bio-information concerning individual cells and subcellular details. One main reason is the simultaneous excitation of high-order modes along with low-order modes, that usually masks the effective information.’](#)

‘Due to higher refractive index, the nucleolus functions as an optical lens, resulting in a notably reduced lasing threshold compared to its surrounding subcellular components (Fig. 1b). Additionally, the separation of gain medium enhanced the threshold difference by 36-fold relative to the gain medium-labeled cells, effectively circumventing emissions with high-order modes (Supplementary Note 1).’

‘Importantly, our approach also enabled nucleolus laser emission from tissue sections, facilitating label-free pathological analysis (Fig. 1e). Furthermore, it allowed the laser emission from suspension cells, where identifying nucleoli using phase contrast imaging alone is challenging. Consequently, we developed the label-free laser-emitting flow cytometry (Fig. 1f). The laser spectral fingerprints can be correlated with the number and size of nucleoli within a single cell, owing to the elimination of high-order modes by the gain separation.’

The following words have been added into the discussion part.

‘Compared to fluorescence imaging, laser emission offers ultrahigh-contrast imaging with minimal background and unique spectrum information.’

‘Instead of labeling cells with fluorescent dyes or micro-nano lasing particles, this study employed subcellular structure with differentiated refractive indices as an indicator to achieve different lasing thresholds within cells. The separation of gain medium with samples further significantly enhanced the threshold difference, leading to label-free selective lasing in nucleolar area. In addition, it enabled the laser emission with primary low-order modes, facilitating the spectrum analysis.’

‘Label-free approach holds paramount importance in the study of subcellular organelles, as it provides a direct means to glean information, particularly concerning the natural kinetics of protein. Nucleolus, as a quintessential model of condensate organelles, exhibits a significant demand for label-free characterization.’

[Comment]

6. Errors. The title in the Supplementary Information is different from that of the main text.

Fig. 2e caption, “inserts” --> “Insets”

Line 217, “the laser emission image result” --> “the laser emission image results”

[response]

Thanks for the reviewer’s comments. We have corrected the mistakes.

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

I appreciate the authors' extensive efforts in addressing the previous comments.

Reviewer #2:

Remarks to the Author:

NCOMMS-24-10382A-Z

In the revised manuscript, the authors have shown in Figure 5 that it is possible to analyze the nucleoli in tissue sections. This demonstrates that the methods developed by the authors can be applied to histopathology, thus resolving a problem that I did not recommend for publication. The validity of the nucleolus data obtained by SLEC in Figure 7 was also confirmed using another nucleolus staining method. Therefore, I recommend that this manuscript be accepted for publication in Nature Communications.

Reviewer #3:

Remarks to the Author:

In the revised manuscript, the authors modify manuscript properly as the reviewers' suggestion. The revised manuscript is convincing satisfactory for the acceptance in Nature Communications.