

Spatial microfluidic holographic integrated platform for label-free and high-dimensional analysis of cancer heterogeneity

Corresponding Author: Dr Ping Su

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The paper presents a quantitative phase microscopy (QPM) system for label-free imaging flow cytometry (IFC) and single-cell analysis via machine learning. IFC is implemented via spatial hydrodynamic focusing microfluidics.

I do not feel that the level of novelty in this paper suits Nature Communications. All elements mentioned above (IFC, hydrodynamic focusing, QPI, machine learning, etc.), and many combinations of them, have been demonstrated in more specialized journals. The paper does not present a breakthrough in the field.

Furthermore, the Abstract is misleading, since it claims for "demonstrating our platform's efficacy for analysing large-scale (>10,000) and highly heterogeneous cell populations". However, in the paper the reader (see line 365 and afterwards) will be disappointed to discover that the acquisition was 3 orders of magnitude(!) slower: "However, compared with traditional commercial flow cytometers, our system's detection throughput is currently constrained ... 30 frames per second (fps)..."

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

Jiayi Shi et al. introduce a Quantitative Phase Microscopy-Image Flow Cytometry (QPM-IFC) system, named the Spatial Microfluidic Holographic Smart (SMHS) platform. This system combines a precisely engineered microfluidic channel, enabling 3D hydrodynamic focusing, with digital holographic microscopy (DHM). This integration enhances the robustness of 3D QPM of cells, allowing for more accurate statistical analysis of cell morphology in a label-free manner. They demonstrate that a set of quantitative phase features, organized hierarchically, achieves high classification accuracy across different cancer cell lines. The technical evaluations are systematic and thorough. However, the novelty of this work is unclear: Is it in the imaging technology, the microfluidic design, the feature extraction methodology, or in presenting a cost-effective approach not previously achieved in QPM and/or IFC?

Main Comments:

1. The authors effectively characterize, validate, and optimize the 3D hydrodynamic focusing conditions (as shown in Fig. 2 and Suppl. Fig. S1-S3). However, the hydrodynamic focusing method seems to follow established concepts, lacking clear innovation.
2. The hardware reflects standard DHM optical configurations, and the phase retrieval/reconstruction processes are well-established. The "Double exposure" technique for noise reduction is not novel, having been used in prior studies (Optics Express 14, 4300 (2006); Sci Rep 9, 9801 (2019)) and in commercial QPI microscopes (e.g., <https://www.lynceetec.com/digital-holography/>).
3. While the estimated throughput is around 1600 particles/sec using beads, the actual DHM imaging throughput demonstrated is approximately 16 cells/sec. This is not particularly impressive compared to state-of-the-art QPI (2D/3D) cytometers.
4. Feature extraction and model training utilize established methods like the Maximum-Relevance and Minimum-Redundancy (MRMR) algorithm. The machine learning models used are standard: LR, KNN, LDA, SVM, NN, DT, Naive

Bayes, Subspace KNN, and Bagged Trees. What is the key advantage of this method?

5. Given point #4, the name "Spatial Microfluidic Holographic Smart" platform may be misleading. The classification relies on standard machine learning models, which may not justify the "smart" label compared to advanced deep learning techniques.

6. The term "pan-cancer" seems overstated. Using multiple cell lines without clinical biopsies from diverse cancer types makes it difficult to support the broad applicability implied by "pan-cancer."

Technical Comments:

1. How did the authors address potential batch effect issues, common in imaging cytometry experiments? This concern is well-documented, for example:

* RxRx1: A Dataset for Evaluating Experimental Batch Correction Methods, DOI: 10.48550/arXiv.2301.05768

* Information-Distilled Generative Label-Free Morphological Profiling Encodes Cellular Heterogeneity, DOI: 10.1002/advs.202307591

2. Is there any attempt to extract the 3D refractive index of the cells in this system?

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

Spatial microfluidic holographic smart platform for label-free and high-dimensional pan-cancer heterogeneity analysis

By

Jiayi Shi, Yanping Liu, Dan Gao, Jianglei Di, Ying Tan, Ping Su, and Jianshe Ma

In this manuscript the authors have combined quantitative phase microscopy (QPM) with imaging flow cytometry (IFC) for single cell analysis using a microfluidic channel. One of the biggest problems with dynamic samples during flow inside a microfluidic channel is defocusing of cells, and the out-of-focus cells cannot be imaged using QPM, leading to inaccurate phase map. The authors could solve this problem and combine system QPM-IFC platform which they called spatial microfluidic holographic smart (SMHS) platform. By means of special design of spatial hydrodynamic focusing microfluidic technique combined with digital holographic microscopy (DHM) they could achieve high-fidelity single-cell QPM imaging. This spatial hydrodynamic focusing microfluidic technique improves 3D cell positioning control to improve QPM measurement accuracy and stability for high-quality imaging. The system is validated using microparticles and biological cells and phase map could be accurately determined. The major application demonstrated is for a variety of cancer cell lines, i.e., breast, lung, colon, cervix and red blood cells. In addition some machine learning algorithms have been utilized.

The proposed QPM-IFC platform could be very useful for early detection of circulating tumor cells with label-free manner. Having the de-focusing problem solved during the flow it could image hundreds of cells in fast manner. The manuscript is well written and experimental results are sufficient to prove the technique supported by details in the supplementary, the paper can be accepted for publication after the following minor corrections.

1. Figure 2 (g) and (f) the hologram is cluttered with speckles and spurious fringes. How much these type of noise sources could affect the accuracy for reconstructing the phase maps? The source of these noises and solving these problems can be found in the following article: "Quantitative Phase Microscopy and Tomography: Techniques using partially spatially coherent monochromatic light, IOP Publishing 2022.

2. Figure 3 (a), healthy red blood cells are generally donut shape, whereas in this figure last column the shapes do not look like donut. What could be the reason?

3. The entire process includes QPM-IFC image recording, phase reconstruction procedure and machine learning algorithms for cell identification. Is it possible to make entire process real time?

4. Quantitative phase microscopy of moving cells and microparticles have been done earlier using waveguide trapping and propulsion "Quantitative phase microscopy of red blood cells during planar trapping and propulsion," Lab on a Chip 18 (19), 3025-3036 (2018). How do you compare your technique with these techniques.

5. In figure 2 (a) and (b) although it is quite clear that focusing of moving particle can be achieved, nevertheless how do you calibrate that the focusing is perfect ~100%.

6. The experimental set-up for QPM is extremely sensitive to external vibrations leading to erroneous phase measurement. How much this configuration will affect phase measurement in the present work? A possible solution for this off-axis configuration is on-axis QPM "Highly stable wide-field common path digital holographic microscope based on a Fresnel biprism interferometer, OSA Continuum 1 (1), 48-55 (2018).

7. How fast is the proposed system for recording and reconstruction of phase maps of live cells of irregular shapes and moving randomly? Can the present system trace nanoscale motion of the fast-moving samples like the results presented in a recent study "Characterizing the consistency of motion of spermatozoa through nanoscale motion tracing, Fertility and Sterility (2024).

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The answers of the authors to my previous remarks made my opinion stronger that the novelty of the paper is unsuitable for Nature Communications. 30fps is not attractive for cell analysis. Much higher throughputs have already been demonstrated by several groups. Hydrodynamic focusing is not the critical issue. I therefore think that the paper is more suitable to biomedical optics or cytometry journals.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

I have read the revised manuscript by Jiayi Shi et al. In general, the authors have addressed most of my comments/concerns, including acknowledging some of the limitations. I think there are a few key points that should be further clarified and revised:

1. In terms of cell/particle focusing, I still find it difficult to understand what's the novelty of the so-called "spatial focusing". The authors claimed that "The findings revealed that spatial focusing is essential for the accurate reconstruction of both amplitude (Fig. 2h, j) and phase (Fig. 2i, k)." But actually, the planar focusing, which is simpler in implementation, can achieve similar accuracy in both amplitude and phase (also seen from Fig. 2h-k). So, I wonder why we need spatial focusing in this case if the planar focusing method already yields the similar performance.

2. Following my comment #1, the authors appear to overlook a large body of recent state-of-the-art demonstrations on particle focusing used together with imaging flow cytometry and related applications. These focusing methods could also be applicable to a wide range of particle sizes, with very impressive focusing precision in 2D plane and/or 3D:

- Flow-rate and particle-size insensitive inertial focusing in dimension-confined ultra-low aspect ratio spiral microchannel, Sensors and Actuators B: Chemical., 132284, 2022
- Dispersion-free inertial focusing (DIF) for high-yield polydisperse micro-particle filtration and analysis, Lab Chip, 2024,24, 4182-4197
- Spiral Large-Dimension Microfluidic Channel for Flow-Rate- and Particle-Size-Insensitive Focusing by the Stabilization and Acceleration of Secondary Flow, Anal. Chem., 96, 4, 1750–1758, 2024

3. I appreciate the effort on adding a new section of experimental demonstrations - cancer cells detection in human blood. In general, I think this proof-of-principle demonstration provide further evidence of how the technique can be used in a scenario closer to clinical setting. In the spike test, the lowest spike ratio is only 1:1000, which is far from the actual CTC application (typically 1:1e5-1e7). Therefore, I would recommend the authors should carefully make their claims - especially in the context of how practical their method can be applied to CTC detection. Also, I think the results in Fig. 7g-j basically showed that the predicted spiked ratio is somewhat consistent with the known ratio. I think without the ground truth of the spiked MDA-MB-231 cells, the authors might not be able to do deeper analysis to quantify the sensitivity and specificity (e.g., ROC analysis). Hence, I would recommend the authors to carefully draw their conclusions. (It seems they didn't experimentally validate the predicted ratio, e.g, Fig. 7g: 507:494).

4. Finally, I still have a problem with the "smart" being in the title, because the machine learning methods are the classical supervised learning ones without any new significant improvements. Even though the authors argue that their Optimized feature selection and machine learning model training strategies are different from others, I find it hard to be convincing to say they are novel. Hence, "smart" in this regard appears to be over claimed. I would highly recommend the authors to reconsider the name.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The authors have improved the manuscript significantly and addressed all the reviewers concern adequately. The authors also addressed the novelty, breakthrough in the manuscript. According to me it is an important work for quantitative cell cytometry and addresses an important aspect in the field, specially quantitative analysis of circulating tumor cells. The manuscript is acceptable for the publication in Nature Communication.

(Remarks on code availability)

Not Required

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have chosen to repeat the points already presented in the paper, and answered very lengthy. This does not change the fact that there is no breakthrough in any of the points raised by the authors. Integration of existing methods to something that was existing already is not, to my opinion, suitable to Nat Comm.

Furthermore, the authors claim for several wrong technical novelties. As a specific example, previous works have shown that phase reconstruction can be done much faster than has been shown by the authors. It was already done at 100fps or even more.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have addressed adequately all my concerns regarding the strengths and limitations of their method. I have one minor suggestion: Supplementary Fig. S11c-d (Distance distribution of microparticles under different focusing states) should be displayed as part of the main-text figure - it shows significant improvement in controlling the particle distribution compared to other methods, worth showing in the main text.

(Remarks on code availability)

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Point-by-point Response to Reviewers' Comments

We thank all the Reviewers for thoroughly examining our manuscript and providing constructive comments. Here, we submit a point-by-point response letter in which we have provided detailed responses to each comment from the Reviewers and made corresponding revisions in our revised manuscript.

Response to Reviewer #1:

Thanks for your comments and approval, which are greatly appreciated.

The response to your comments is list below:

The paper presents a quantitative phase microscopy (QPM) system for label-free imaging flow cytometry (IFC) and single-cell analysis via machine learning. IFC is implemented via spatial hydrodynamic focusing microfluidics.

1. All elements mentioned above (IFC, hydrodynamic focusing, QPI, machine learning, etc.), and many combinations of them, have been demonstrated in more specialized journals. The paper does not present a breakthrough in the field.

Response: Thanks a lot. We appreciate your valuable feedback, and fully understand the concerns regarding the novelty and significance of our research. Indeed, as you rightly pointed out, technologies such as quantitative phase microscopy (QPM), hydrodynamic focusing, machine learning, and some of their combinations, have already been demonstrated in other journals. However, we believe that our study offers unique value and innovation in the following aspects, which align with the interdisciplinary and high-impact research criteria that Nature Communications values.

1) Integrated innovation and precise control

Our research places a strong emphasis on integrated innovation and precise system control. For the first time, we have optimized and combined single-cell microfluidics technology based on spatial hydrodynamic focusing with digital holographic microscopy (DHM) to achieve high-performance and label-free single-cell analysis. By adjusting the flow rate ratio of sheath flow to sample flow in the microfluidics module, cells or particles of different properties and sizes can be precisely focused in both vertical and horizontal directions within the detection area. By aligning the recording focal plane of DHM with the spatial

hydrodynamic focusing plane of the sample flow, we can directly obtain clear and precise focused single-cell hologram. This level of precision has not been achieved in previous studies. As illustrated in our manuscript introduction (see lines 62-70), prior researches¹⁻⁶ have not made great effort to achieve strict axial constraints, leading to issues such as cell floating and overlapping, which affect the accuracy of single-cell identification, and hinder the automation and standardization of the detection process.

2) Technological breakthroughs and performance enhancement

Thanks to the aforementioned integrated innovation, we have significantly simplified the precise single-cell quantitative phase reconstruction process in QPM-IFC. We directly capture the hologram of focused single cells and particles, eliminating the need for digital refocusing or any other compensation. Consequently, we have greatly reduced the reconstruction time for single-cell quantitative phase microscopy images, saving computational resources. Our system completes the reconstruction of a single-cell hologram in approximately 0.34 seconds using lab-written MATLAB codes on a laptop equipped with a 2.8 GHz Intel Core i7 CPU and 16 GB of RAM. This is significantly faster compared to typical process that requires digital refocusing, which takes about 7 seconds (see lines 133-137 of the revised manuscript, lines 330-354 of the revised Supplementary Note 3, and newly added Supplementary Table 5). This represents a technological breakthrough in image processing and reconstruction in QPM-IFC, with clear advantages in the analysis of large-scale cell populations.

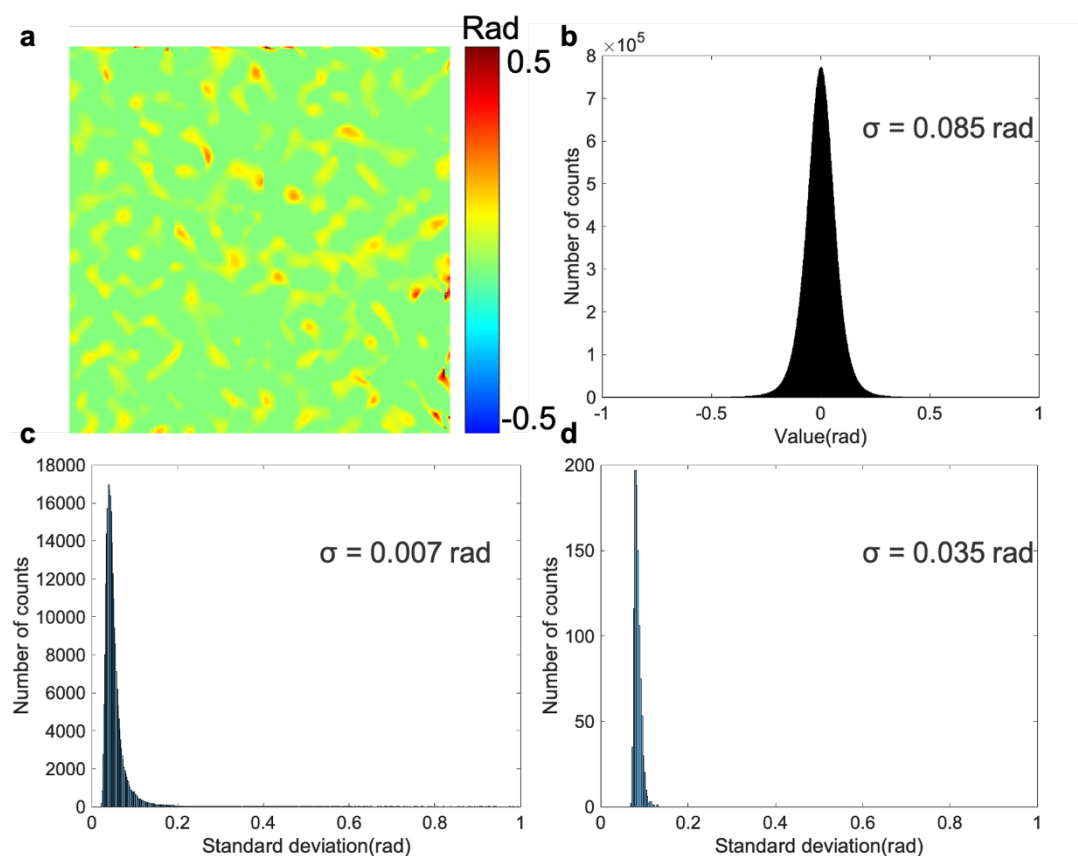
Supplementary Table 5. Comparison of QPM phase reconstruction workflow and speed

QPM phase reconstruction workflow in our research					
Frequency filtering	Numerical reconstruction	Phase extraction	Phase unwrapping	Phase correction	Total
0.084 s	0.009 s	0.004 s	0.056 s	0.189 s	0.343 s
QPM phase reconstruction workflow in other research ^{14*}					
Holographic demodulation	Numerical refocusing	Phase extraction	Aberration compensation	Denoising &Unwrapping	Total
0.06 s	4.46 s	0.01 s	0.42 s	2.76 s	7.71 s

*Supplementary Reference 14. Pirone, D. et al. Speeding up reconstruction of 3D tomograms in holographic flow cytometry via deep learning. *Lab. Chip* **22**, 793-804 (2022).

Furthermore, we have added a test for system noise stability (see lines 120-127 of the revised manuscript, lines 228-267 of the revised Supplementary Note 2, and newly added

Supplementary Fig. 7). The standard deviation of temporal phase noise (0.035 rad) is significantly lower than that of the typical Mach-Zehnder configuration in DHM (0.2 rad), highlighting the superiority of our system over other DHMs. The standard deviation of spatial phase noise (0.007 rad) is much lower than the average phase height of typical cells (3-5 rad), ensuring minimal impact on measurements. This excellent stability provides a solid foundation for capturing clear holograms of in-flow single particles or cells, which is key to achieving high-quality imaging and data acquisition.



Supplementary Fig. 7 Noise measurement of our DHM. a Spatial noise distribution of background at first frame. **b** Spatiotemporal noise histogram of 1,000 frames in approximately 2.8 h time-series. **c** Histogram of the standard deviation of spatial noise. **d** Histogram of the standard deviation of temporal noise.

3) Optimized feature selection and machine learning model training strategies

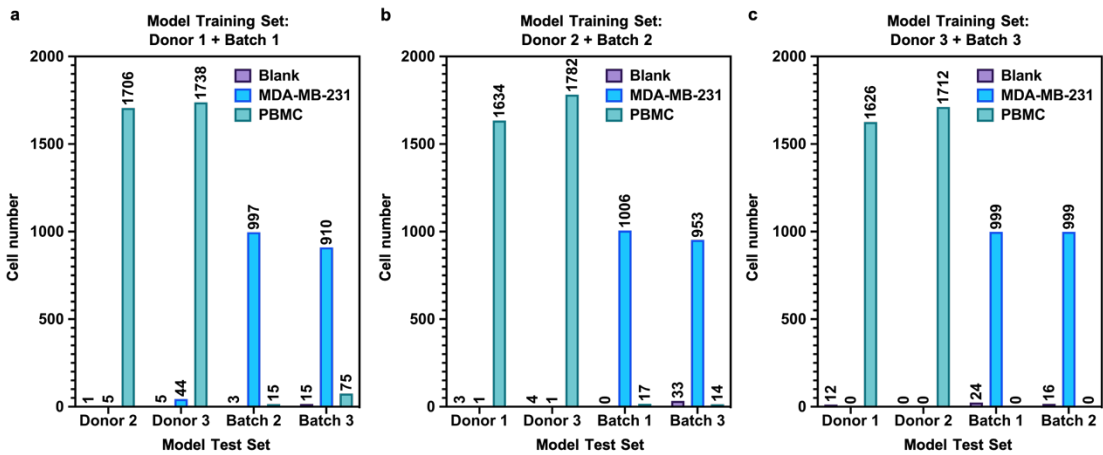
While training models with the full set of QPM features we extracted has already achieved a rather satisfactory accuracy rate (see Supplementary Table 7, 8, 10 and 11), we did not stop there. We consider the correlation and redundancy of QPM features more comprehensively, performing predictive performance screening to obtain the optimal feature dataset. Notably, we

construct an incremental feature selection (IFS) strategy using the Maximum-Relevance and Minimum-Redundancy (MRMR) feature ranking algorithm based on mutual information, which is innovative and has shown significant effectiveness in training 9 classic machine learning models, proving the effectiveness of this strategy. Training models based on this strategy can largely shorten training time, increase prediction speed, and obtain the highest classification accuracy with as few features as possible (see Supplementary Table 9 and 12).

We have supplemented the proof of the model strategy's generalization ability (see lines 382-386 of the revised manuscript; newly added Supplementary Note 8, Supplementary Table 13 and Supplementary Fig. 19-22). Notably, we meticulously considered and analyzed the impact of batch effects, performing cross-validation used in relevant literatures^{7, 8}. The results demonstrated high cross-validation accuracy, proving the generalizability and broad applicability of our LDA model post-training with the MRMR-IFS strategy.

Supplementary Table 13. Cross-validation results with LDA models

Training Dataset			Test Dataset			Test Dataset		
			PBMC			MDA-MB-231		
Blank	PBMC	MDA-MB-231	Donor 1	Donor 2	Donor 3	Batch 1	Batch 2	Batch 3
Blank	Donor 1	Batch 1	NA	99.6%	97.3%	NA	98.2%	91.0%
Blank	Donor 2	Batch 2	99.8%	NA	99.7%	98.3%	NA	95.3%
Blank	Donor 3	Batch 3	99.3%	100.0%	NA	97.7%	98.4%	NA



Supplementary Fig. 22 Cross-validation results with LDA models. Models are trained on (a) Donor 1's PBMC with Batch 1's MDA-MB-231, (b) Donor 2's PBMC with Batch 2's MDA-MB-231, and (c) Donor 3's PBMC with Batch 3's MDA-MB-231, with remaining batches and donors serving as the test set in a rotating fashion. The bars represent the predicted outcomes

for different cell types within the test set, including Blank (purple bars), MDA-MB-231 (blue bars), and PBMC (green bars).

4) High accuracy verification based on heterogeneous cell population and broad application prospects

A remarkable >99.9% prediction accuracy has been achieved across multiple cancer cell types, breast cancer subtypes, and blood cells (see lines 379-382, and newly added Figure 7 of the revised manuscript for our latest research), demonstrating its exceptional effectiveness in analyzing highly heterogeneous cell populations. Notably, this system also exhibits high accuracy in the analysis of simulated clinical blood samples (see lines 386-392, and newly added Figure 7 of the revised manuscript), highlighting its great potential for clinical applications.

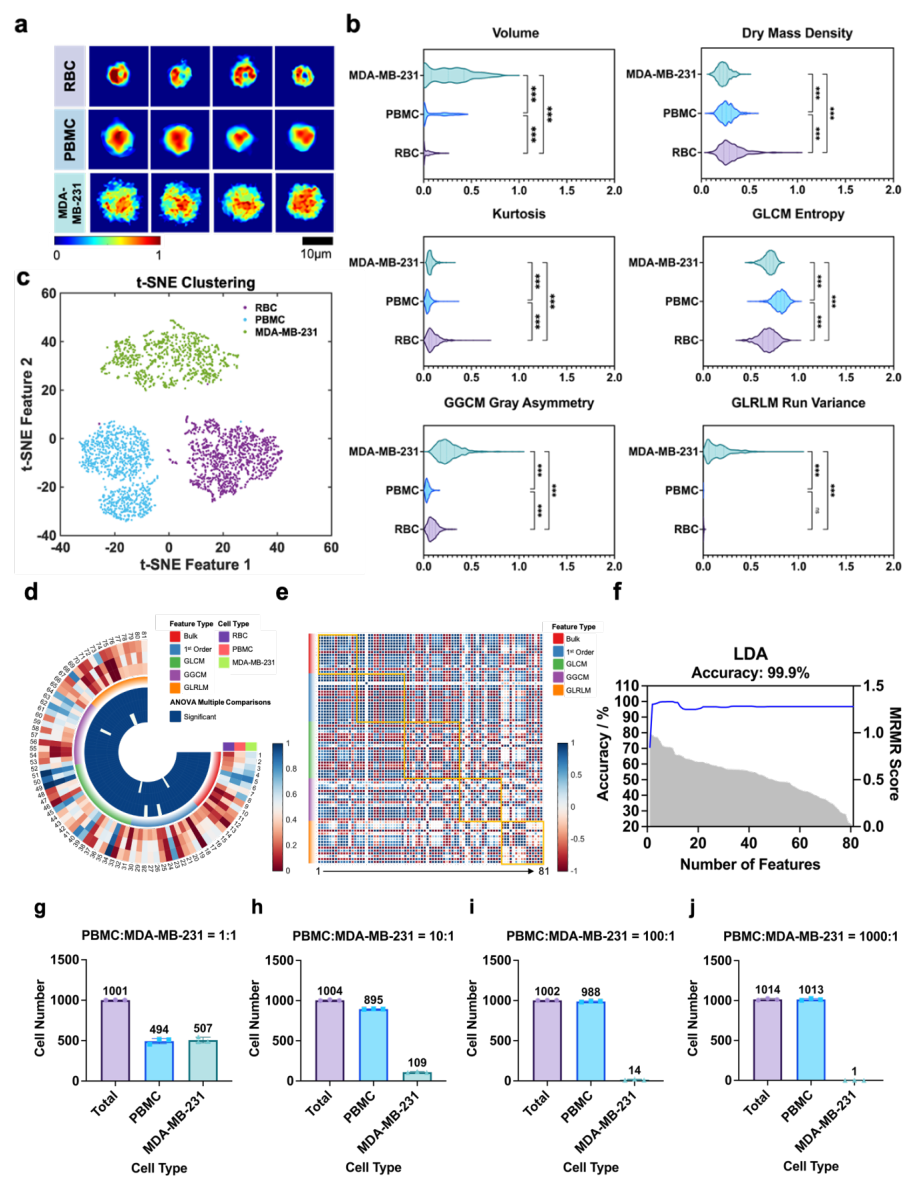


Fig. 7 Identification and enumeration of breast cancer cells in human blood. (a) Single-cell QPM phase maps of red blood cells (RBCs), peripheral blood mononuclear cells (PBMCs), and MDA-MB-231 cells. The color bar denotes the scale of normalized optical path difference (OPD) to reflect phase information (Scale bars: 10 μm). (b) Violin plots of 6 QPM phase features ($N = 1,000$ for each type), including volume (Bulk), dry mass density (Bulk), kurtosis (First-order histogram), entropy (GLCM), gray asymmetry (GGCM), and run variance (GLRLM). *** indicates $p\text{-value} < 0.001$, ns indicates $p\text{-value} > 0.05$, based on ordinary one-way ANOVA pairwise comparisons (Tukey's test). (c) t-SNE plot of RBCs (purple dots), PBMCs (blue dots), MDA-MB-231 cells (green dots) ($N = 1,000$ for each type). In (d), the outer circular heatmap displays the mean value distribution of 81 features across RBCs, PBMCs and MDA-MB-231 cells. The color bar denotes the scale of normalized mean values ($N = 1,000$ for each type). The inner binary heatmap shows the results of ordinary one-way ANOVA pairwise comparisons (Tukey's test). Dark blue visualizes whether these differences are statistically significant. The significance level (α) is set to 0.05. (e) Pearson correlation matrix of 81 QPM phase features visualized as heatmap. Blue indicates positive correlation, while red indicates negative correlation. Different feature subsets are divided by yellow boxes. The color bar represents the scale of Pearson correlation coefficient. (f) Incremental feature selection (IFS) curves for LDA model trained for classifying PBMCs and MDA-MB-231 cells, with the highest predictive accuracy labeled accordingly. A total of PBMCs ($N = 5,137$) and MDA-MB-231 cells ($N = 3,040$) data were used for training. The gray shadow represents the MRMR scores of features. The blue curve illustrates the accuracy rates on the ten-fold cross-validation set. (g) to (j) illustrate the predicted total cell numbers (purple bars), PBMC numbers (blue bars), and MDA-MB-231 cell numbers (green bars) in PBMC samples spiked with MDA-MB-231 cells by the LDA model, with the ratios of PBMC to MDA-MB-231 are 1:1 (g), 10:1 (h), 100:1 (i), and 1,000:1 (j). The numbers on top of each bar represents the mean of 3 independent replicates. Source data are provided as a Source Data file.

We believe that these unique values and innovations meet the publication criteria for high-impact research in Nature Communications. We look forward to your further guidance and suggestions to better showcase the value of our research. Once again, we appreciate your valuable time and professional opinions.

2. Furthermore, the Abstract is misleading, since it claims for "demonstrating our platform's efficacy for analysing large-scale (>10,000) and highly heterogeneous cell populations". However, in the paper the reader (see line 365 and afterwards) will be disappointed to discover that the acquisition was 3 orders of magnitude(!) slower: "However, compared with traditional commercial flow cytometers, our system's detection throughput is currently constrained ... 30 frames per second (fps)..."

Response: Thanks a lot. We apologize for the confusion. We would like to clarify the distinction between the scale of cell populations analyzed and the detection throughput of our platform. The number of "10,000" cells mentioned in the Abstract is the total volume of single-cell dataset we currently used in the machine learning modeling and forecasting, not the speed at which individual cells are imaged and classified. The 30 frames per second (fps) mentioned in the paper (see line 436 of the revised manuscript) refers to the CMOS camera's acquisition rate, which is indeed a current limitation of our system. We agree that this detection throughput is lower compared to traditional commercial flow cytometers, and we have been transparent about this constraint in our manuscript. We acknowledge that our SMHS platform is still in the early stage of proof-of-concept. The detection throughput, while a significant consideration, is not the primary focus at this stage of development. We are fully aware that for practical applications, the throughput will need to be enhanced, and we have plans in place to address this by modifying the system configuration, including using cameras with faster frame rates (in the thousands to millions) to increase the throughput in future iterations. We appreciate your feedback, and we have revised the Abstract to avoid any further confusion (see lines 26-27 of the revised manuscript).

Response to Reviewer #2:

Thanks for your comments and approval, which are greatly appreciated.

The response to your comments is list below:

1. Jiayi Shi et al. introduce a Quantitative Phase Microscopy-Image Flow Cytometry (QPM-IFC) system, named the Spatial Microfluidic Holographic Smart (SMHS) platform. This system combines a precisely engineered microfluidic channel, enabling 3D hydrodynamic focusing, with digital holographic microscopy (DHM). This integration enhances the robustness of 3D QPM of cells, allowing for more accurate statistical analysis of cell morphology in a label-free manner. They demonstrate that a set of quantitative phase features, organized hierarchically, achieves high classification accuracy across different cancer cell lines. The technical evaluations are systematic and thorough. However, the novelty of this work is unclear: Is it in the imaging technology, the microfluidic design, the feature extraction methodology, or in presenting a cost-effective approach not previously achieved in QPM and/or IFC?

Response: We appreciate your thoughtful comments, which have guided us in clarifying the novelty and significance of our research. We believe that the most significant highlight of our study lies in the fact that, while many of the technologies involved, including digital holographic microscopy (DHM), microfluidics, and machine learning, are not entirely new, the essence of our innovation is in the synergistic combination and optimization of these different technological modules. This integration has achieved performance and accuracy levels that have not been reported before, aligning with Nature Communications' encouragement of interdisciplinary and cross-innovative research. Next, we would like to reiterate the innovative aspects of this research to you.

1) Integrated innovation and precise control

Our research places a strong emphasis on integrated innovation and precise system control. For the first time, we have optimized and combined single-cell microfluidics technology based on spatial hydrodynamic focusing with DHM to achieve high-performance and label-free single-cell analysis. By adjusting the flow rate ratio of sheath flow to sample flow in the microfluidics module, cells or particles of different properties and sizes can be precisely focused in both vertical and horizontal directions within the detection area. By

aligning the recording focal plane of DHM with the spatial hydrodynamic focusing plane of the sample flow, we can directly obtain clear and precise focused single-cell hologram. This level of precision has not been achieved in previous studies. As illustrated in our manuscript introduction (see lines 62-70), prior researches¹⁻⁶ have not made great effort to achieve strict axial constraints, leading to issues such as cell floating and overlapping, which affect the accuracy of single-cell identification, and hinder the automation and standardization of the detection process.

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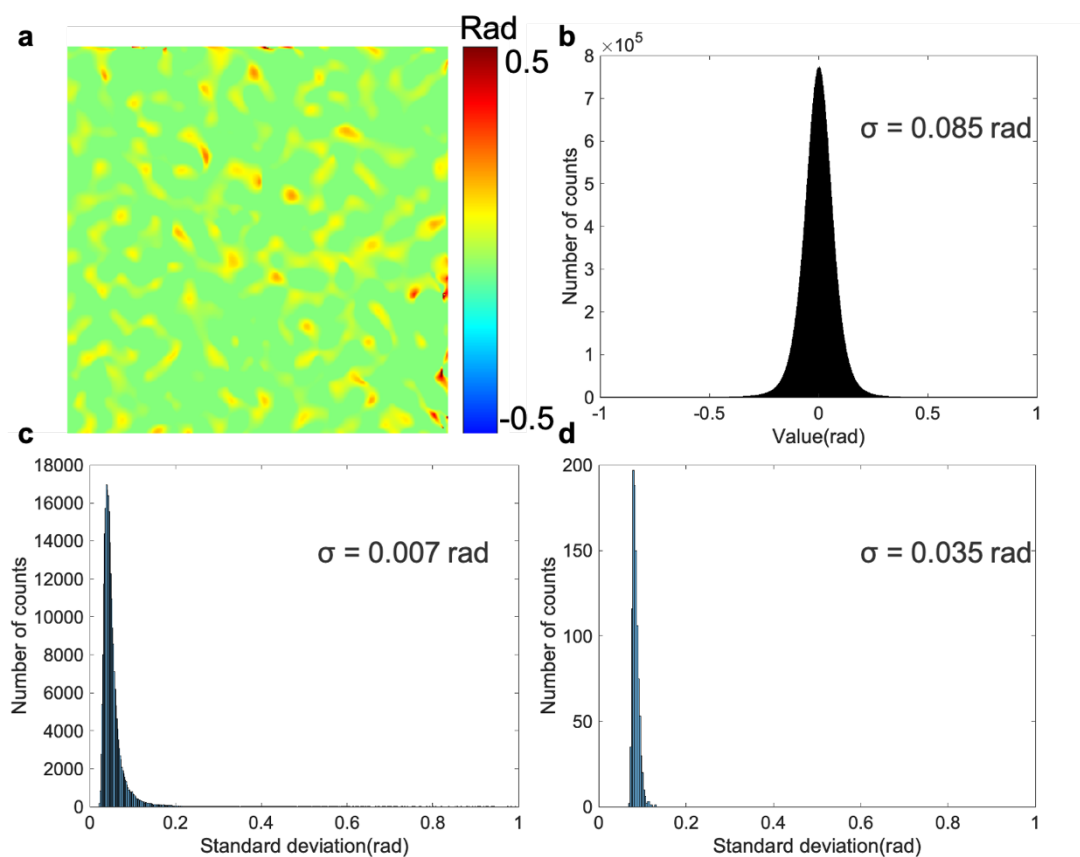
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Supplementary Fig. 7 Noise measurement of our DHM. **a** Spatial noise distribution of background at first frame. **b** Spatiotemporal noise histogram of 1,000 frames in approximately 2.8 h time-series. **c** Histogram of the standard deviation of spatial noise. **d** Histogram of the standard deviation of temporal noise.

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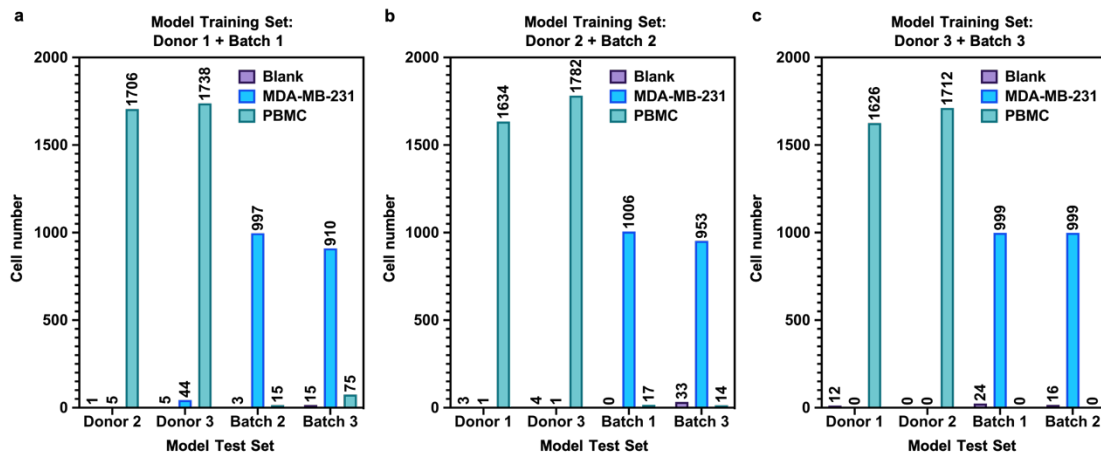
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there. We consider the correlation and redundancy of QPM features more comprehensively, performing predictive performance screening to obtain the optimal feature dataset. Notably, we construct an incremental feature selection (IFS) strategy using the Maximum-Relevance and Minimum-Redundancy (MRMR) feature ranking algorithm based on mutual information, which is innovative and has shown significant effectiveness in training 9 classic machine learning models, proving the effectiveness of this strategy. Training models based on this strategy can largely shorten training time, increase prediction speed, and obtain the highest classification accuracy with as few features as possible (see Supplementary Table 9 and 12).

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			PBMC			MDA-MB-231		
Blank	PBMC	MDA-MB-231	Donor 1	Donor 2	Donor 3	Batch 1	Batch 2	Batch 3
Blank	Donor 1	Batch 1	NA	99.6%	97.3%	NA	98.2%	91.0%
Blank	Donor 2	Batch 2	99.8%	NA	99.7%	98.3%	NA	95.3%
Blank	Donor 3	Batch 3	99.3%	100.0%	NA	97.7%	98.4%	NA



Supplementary Fig. 22 Cross-validation results with LDA models. Models are trained on (a) Donor 1's PBMC with Batch 1's MDA-MB-231, (b) Donor 2's PBMC with Batch 2's MDA-MB-231, and (c) Donor 3's PBMC with Batch 3's MDA-MB-231, with remaining batches and donors serving as the test set in a rotating fashion. The bars represent the predicted outcomes for different cell types within the test set, including Blank (purple bars), MDA-MB-231 (blue bars), and PBMC (green bars).

4) High accuracy verification based on heterogeneous cell population and broad application prospects

A remarkable >99.9% prediction accuracy has been achieved across multiple cancer cell types, breast cancer subtypes, and blood cells (see lines 379-382 and newly added Figure 7 of the revised manuscript for our latest research), demonstrating its exceptional effectiveness in analyzing highly heterogeneous cell populations. Notably, this system also exhibits high accuracy in the analysis of simulated clinical blood samples (see lines 386-392, and newly added Figure 7 of the revised manuscript), highlighting its great potential for clinical applications.

Once again, we appreciate your valuable feedback and the opportunity to clarify the contributions of our research. In the following letter, we have provided detailed responses to each comment you raised. We are also open to receiving any further feedback you may have.

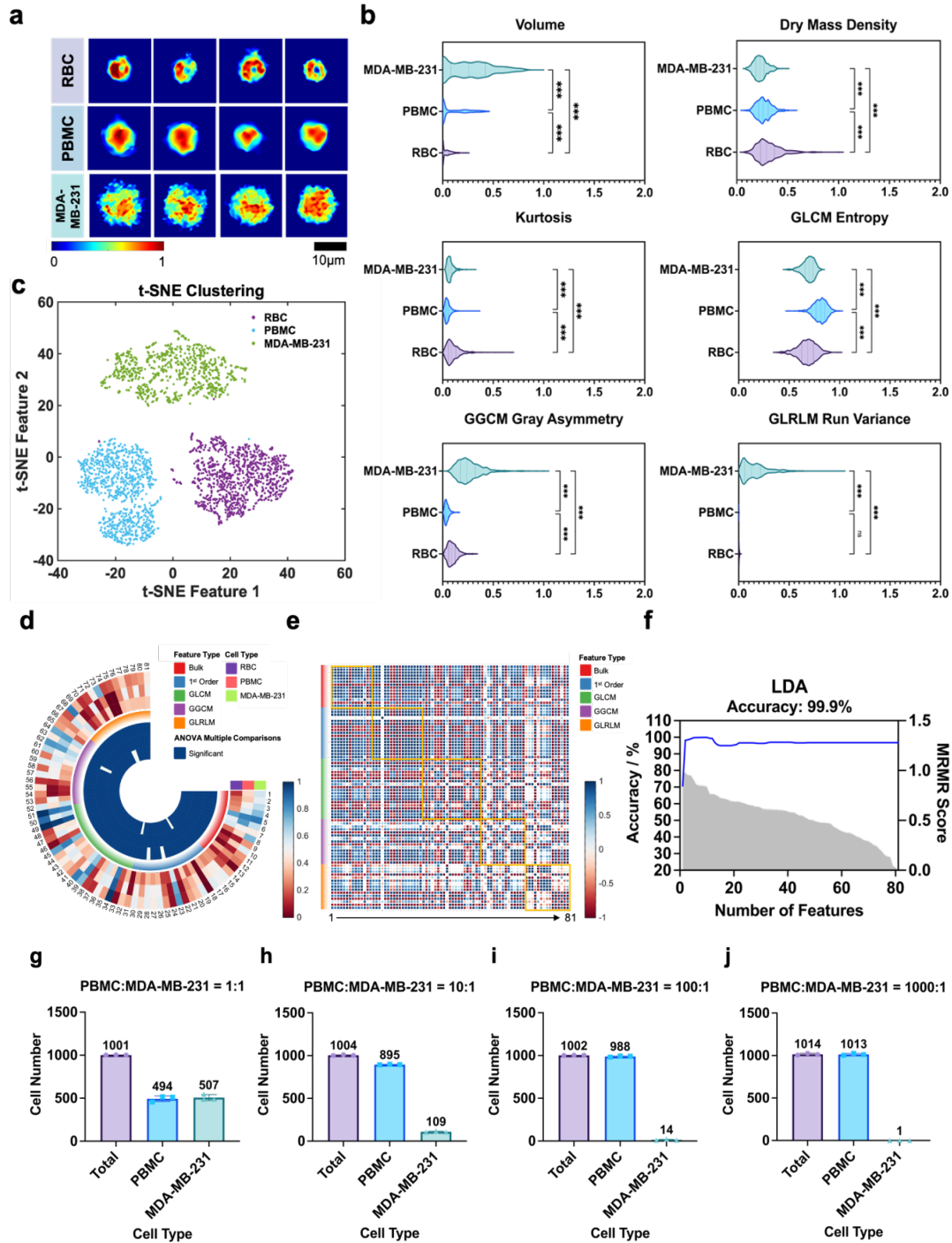


Fig. 7 Identification and enumeration of breast cancer cells in human blood. (a) Single-cell QPM phase maps of red blood cells (RBCs), peripheral blood mononuclear cells (PBMCs), and MDA-MB-231 cells. The color bar denotes the scale of normalized optical path difference (OPD) to reflect phase information (Scale bars: 10 μ m). (b) Violin plots of 6 QPM phase features ($N = 1,000$ for each type), including volume (Bulk), dry mass density (Bulk), kurtosis (First-order histogram), entropy (GLCM), gray asymmetry (GGCM), and run variance (GLRLM). *** indicates p -value < 0.001 , ns indicates p -value > 0.05 , based on ordinary one-way ANOVA pairwise comparisons (Tukey's test). (c) t-SNE plot of RBCs (purple dots),

PBMCs (blue dots), MDA-MB-231 cells (green dots) ($N = 1,000$ for each type). In (d), the outer circular heatmap displays the mean value distribution of 81 features across RBCs, PBMCs and MDA-MB-231 cells. The color bar denotes the scale of normalized mean values ($N = 1,000$ for each type). The inner binary heatmap shows the results of ordinary one-way ANOVA pairwise comparisons (Tukey's test). Dark blue visualizes whether these differences are statistically significant. The significance level (α) is set to 0.05. (e) Pearson correlation matrix of 81 QPM phase features visualized as heatmap. Blue indicates positive correlation, while red indicates negative correlation. Different feature subsets are divided by yellow boxes. The color bar represents the scale of Pearson correlation coefficient. (f) Incremental feature selection (IFS) curves for LDA model trained for classifying PBMCs and MDA-MB-231 cells, with the highest predictive accuracy labeled accordingly. A total of PBMCs ($N = 5,137$) and MDA-MB-231 cells ($N = 3,040$) data were used for training. The gray shadow represents the MRMR scores of features. The blue curve illustrates the accuracy rates on the ten-fold cross-validation set. (g) to (j) illustrate the predicted total cell numbers (purple bars), PBMC numbers (blue bars), and MDA-MB-231 cell numbers (green bars) in PBMC samples spiked with MDA-MB-231 cells by the LDA model, with the ratios of PBMC to MDA-MB-231 are 1:1 (g), 10:1 (h), 100:1 (i), and 1,000:1 (j). The numbers on top of each bar represents the mean of 3 independent replicates. Source data are provided as a Source Data file.

Main Comments:

1. The authors effectively characterize, validate, and optimize the 3D hydrodynamic focusing conditions (as shown in Fig. 2 and Suppl. Fig. S1-S3). However, the hydrodynamic focusing method seems to follow established concepts, lacking clear innovation.

Response: Thank you for acknowledging the work we have done to characterize and optimize the spatial hydrodynamic focusing conditions. Indeed, we agree that the technology of hydrodynamic focusing is not new. **However, we would like to clarify that while our method is based on the established concept of hydrodynamic focusing, our implementation is distinct and specifically designed for this task. To the best of our knowledge, this combination of spatial hydrodynamic focusing and DHM is being proposed for the first time.**

We summarized the current advanced QPM-IFC technology (see Supplementary Table 14). Single-cell microfluidics technology based on strict spatial hydrodynamic focusing has not been applied in the research field of QPM-IFC, which we believe is worth emphasizing again. Compared to other single-cell microfluidics techniques, the advantage of spatial hydrodynamic focusing technology is that it does not require additional physical fields, or complex channel

structure design and fabrication. It can flexibly adjust the focusing effect, thus being adaptable to the detection of single cells/particles of different sizes and properties, which perfectly matches the design requirements of our platform. Therefore, this integration is not merely a reiteration of established concepts, but a significant advancement of using spatial hydrodynamic focusing technology for label-free single-cell analysis in practice.

Furthermore, although we have drawn upon past studies in the design of our hydrodynamic microfluidic chip, we have tailored the key parameters of hydrodynamics focusing to meet the specific requirements of our platform, specifically the flow rate ratios of each sheath flow to sample flow (see Figure 2 and Supplementary Figure 1-3). By adjusting these ratios, we not only achieve spatial focusing of individual cells but, more importantly, the focused sample flow is strictly confined to the center of the channel. Therefore, by aligning the recording plane of DHM with the spatial focusing plane of the sample flow, we can directly obtain the holograms of single cells in focus. This is why we specifically define spatial hydrodynamic focusing (see line 109 and afterwards of the revised manuscript) to distinguish our work from the general 3D hydrodynamic focusing setup.

We hope this clarification addresses your concerns regarding the novelty of our research. We appreciate your time and consideration and remain open to any further suggestions or feedback you may have.

2. The hardware reflects standard DHM optical configurations, and the phase retrieval/reconstruction processes are well-established. The "Double exposure" technique for noise reduction is not novel, having been used in prior studies (Optics Express 14, 4300 (2006); Sci Rep 9, 9801 (2019)) and in commercial QPI microscopes (e.g., <https://www.lynceetec.com/digital-holography/>).

Response: Indeed, our platform employs the standard DHM equipment and the well-established phase retrieval/reconstruction techniques, including the "double exposure" method for noise reduction. What we would like to clarify is that, although these components are not individually novel, they form the foundation of our platform's excellent performance.

One of the key advantages of using DHM to obtain phase information is its high stability. We have conducted tests that demonstrate the exceptional spatiotemporal stability of our cage-structured DHM system (see lines 120-127 of the revised manuscript, lines 228-267 of the

revised Supplementary Note 2, and newly added Supplementary Fig. 7). This stability is vital for obtaining clear holograms of in-flow single particles or cells, ensuring high-quality imaging and data acquisition. Furthermore, by integrating DHM with spatial hydrodynamic focusing, we have achieved precise hologram acquisition and rapid phase reconstruction without the need for the time-consuming and labor-intensive digital refocusing process. This integration significantly enhances the efficiency of phase reconstruction (see lines 133-137 of the revised manuscript, lines 330-354 of the revised Supplementary Note 3, and newly added Supplementary Table 5), which not only streamlines the process but also gives new vitality to the innovative application of the mature DHM.

We are grateful for your question and for the opportunity to further clarify the technology of our research. We have reorganized the descriptions regarding DHM in the Abstract (see lines 20-21), Introduction (see line 89) and Discussions (see lines 401-407) of the revised manuscript. We have also added the reference you provided next to the relevant technical details of the "double exposure" technique in our manuscript (see line 515) and the revised Supplementary Note 3 (see line 280).

3. While the estimated throughput is around 1600 particles/sec using beads, the actual DHM imaging throughput demonstrated is approximately 16 cells/sec. This is not particularly impressive compared to state-of-the-art QPI (2D/3D) cytometers.

Response: We would like to thank you for your critical and constructive comments. As mentioned in our Discussions (see lines 432-435 of the revised manuscript), the estimated throughput of our microfluidics module has achieved around 1,600 particles/sec using beads, which is close to the level of commercial IFC systems⁹. However, the detection throughput is currently limited by the camera's capture frame rate of only 30 fps. We have been transparent about this constraint in our manuscript, acknowledging that our SMHS platform is still in the early stage of proof-of-concept. The detection throughput, while a significant consideration, is not the primary focus at this stage of development. We are fully aware that for practical applications, the throughput will need to be enhanced, and we have plans in place to address this by modifying the system configuration, including using cameras with faster frame rates (in the thousands to millions) to increase the throughput in future iterations. Unfortunately, due to time and financial constraints, we have not been able to complete the upgrade of the camera,

so we refined the limitations in our Discussions (see lines 430-440 of the revised manuscript). Thank you very much for pointing out this important issue, which provides a direction for our next research.

4. Feature extraction and model training utilize established methods like the Maximum-Relevance and Minimum-Redundancy (MRMR) algorithm. The machine learning models used are standard: LR, KNN, LDA, SVM, NN, DT, Naive Bayes, Subspace KNN, and Bagged Trees. What is the key advantage of this method?

Response: We appreciate your interest in understanding the key advantages of our feature selection and model training approach. While it is true that we have utilized established methods such as the MRMR algorithm and standard machine learning models, our approach offers several distinct advantages that contribute to the novelty and effectiveness of our study.

Our use of the MRMR algorithm is not merely as a standalone feature selection tool but as part of an innovative MRMR-IFS strategy. This strategy allows us to dynamically select the most relevant features while iteratively reducing redundancy. The most critical advantage of this strategy is constructing the most streamlined and effective combination of features for model training, therefore significantly reduces the time required for model training, enhances the speed of prediction and saves computing resources. As shown in Figure R1, we can see that more features mean slower prediction speeds (a) and longer training times (b). However, from the analysis in Figure 4 and 6, when the number of features reaches a certain threshold, the accuracy of model predictions will no longer increase, and may even decline. Therefore, by selecting the most relevant features with the least redundancy, we are able to achieve the highest classification accuracy with as few features as possible. This not only simplifies our models but also reduces overfitting, leading to more generalizable and robust predictions, which is important especially when dealing with large datasets. The effectiveness of our MRMR-IFS strategy is further highlighted by its success across 9 different classic machine learning models. This broad applicability demonstrates the versatility and strength of our approach.

In summary, while the components of our method may be individually known, our innovative combination and application of these methods result in a feature selection and model training process that is more efficient, faster, and accurate. We believe these advantages position our study at the forefront of leveraging QPM features for high-performance cell analysis. We hope

this response addresses your question and highlights the key innovative aspects of our approach, which we have made corresponding summary in our Discussions section (see lines 425-427 of the revised manuscript).

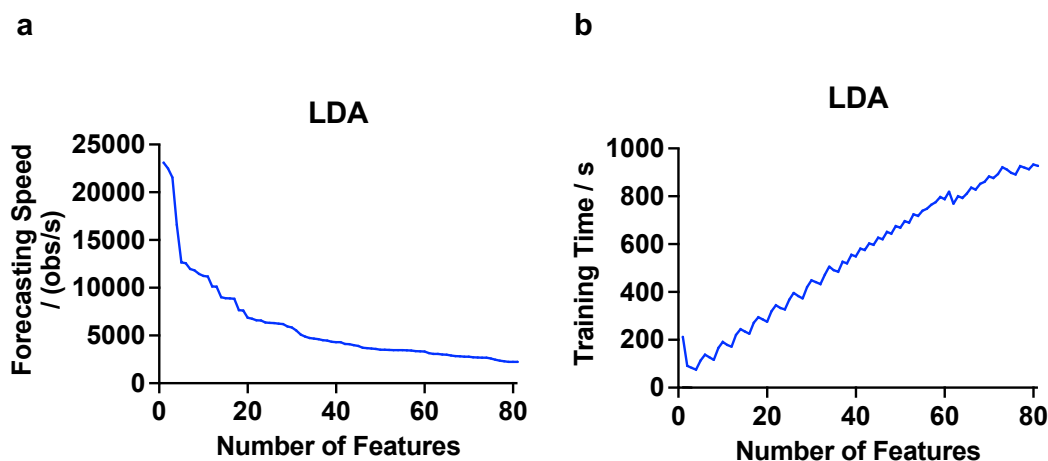


Figure R1. **a** The forecasting speed of LDA model used for multiple cancer cell types classification. **b** The training time of LDA model used for multiple cancer cell types classification.

5. Given point #4, the name "Spatial Microfluidic Holographic Smart" platform may be misleading. The classification relies on standard machine learning models, which may not justify the "smart" label compared to advanced deep learning techniques.

Response: We appreciate your feedback and understand your concerns about the use of the term "smart" in the name of our platform. We would like to address these concerns directly and provide a clear explanation of the rationality of our current approach, the intelligent aspects of our platform and our future directions.

Firstly, we acknowledge the potential for the term "smart" to be misconstrued in the context of our current technology, because it may imply the use of cutting-edge deep learning techniques. However, in this work, our primary aim is to validate our platform's ability to efficiently analyze and classify cell populations based on the QPM features and automated discrimination algorithms. We would like to explain that, despite utilizing standard machine learning models, our platform already demonstrates significant advantages and elements of intelligence. We have automated feature selection and streamlined the model training process, which can be indicative of the smart system efficiency. Our platform employs the MRMR feature ranking algorithm

based on mutual information, which intelligently selects the most informative features, reducing training time and increasing prediction speed. Moreover, we have achieved a classification prediction accuracy of $\geq 99.9\%$ across various cell types, showcasing its ability to make precise and intelligent decisions in cellular analysis.

Besides, the scalability and adaptability of our platform are key to its intelligence. The platform's design allows for easy integration of more advanced artificial intelligence (AI) models, which can further enhance its level of smart operation and analytical capabilities. Given the relatively simple sample environment, our choice of standard machine learning models has proven to be sufficient for achieving high accuracy in discrimination. However, we acknowledge that in more complex sample analyses, feature extraction becomes more challenging, necessitating the use of advanced AI models for precise analysis and to further deepen the "smart" aspect of our platform. Advanced AI models such as Convolutional Neural Networks (CNNs)¹⁰, Recurrent Neural Networks (RNNs)¹¹, and Residual Network (ResNet)¹²,¹³ have shown promise. As we move forward, we plan to integrate more advanced AI technologies to enhance our platform's analytical capabilities. Therefore, we have also included this as an important direction for future development in the Discussions section of our revised manuscript (see lines 440-445).

In conclusion, we believe that our platform will offer greater flexibility, integration, and intelligence in future updates and upgrades. After careful consideration, we have decided to retain the term "smart" to reflect the powerful advantages of our platform in feature extraction and classification accuracy.

6. The term "pan-cancer" seems overstated. Using multiple cell lines without clinical biopsies from diverse cancer types makes it difficult to support the broad applicability implied by "pan-cancer."

Response: We appreciate your feedback on the use of the term "pan-cancer" in our manuscript. In response to your concern, we have taken the following steps to address the issue.

First, we have revised the manuscript to replace the term "pan-cancer" with more accurate descriptors that reflect the scope of our study, like "multiple cancer cell types" or "multi-cancer" to more accurately convey the range of cell lines included in our analysis.

Secondly, we fully recognize the significance of validating our platform's broad applicability using real/clinical samples to deepen our research. However, due to time constraints and challenges in obtaining clinical samples and constructing models, we have followed the methodologies of several previous studies¹⁴⁻¹⁶, utilizing simulated circulating tumor cells (CTCs) samples for the label-free detection of cancer cells in human blood. These references have provided a solid foundation for our research design. Specifically, we procured blood from three healthy volunteers, and isolated red blood cells (RBCs) and peripheral blood mononuclear cells (PBMCs). Aligning with the practical approach of CTCs detection, enriching CTCs is necessary due to their rarity. According to the previous references, RBCs removal by centrifugation or lysis is a common step in CTC isolation to minimize interference. We then simulated CTCs blood samples by mixing PBMCs with MDA-MB-231 cell suspensions, with the ratios of PBMC to MDA-MB-231 ranging from 1:1 to 1000:1. The results illustrate the high consistency between the classification ratios predicted by the LDA model and the actual mixing ratios of the samples, validating our SMHS platform's potential to identify CTCs in human blood samples and accurately count them, thereby highlighting its application potential in clinical settings. We have added a new section titled "Identification and enumeration of breast cancer cells in human blood" in our revised manuscript (see lines 352-392 and newly added Figure 7 of the revised manuscript). We kindly invite you to review this section.

Third, we have also included a section on future directions in the Discussions part of our manuscript (see lines 449-458 of the revised manuscript), outlining our plans to expand the study to include clinical biopsies from a wider range of cancer types. This will be crucial in validating the "pan-cancer" applicability of our platform and is a necessary step towards realizing its full potential.

We understand the importance of using precise and appropriate terminology, especially when discussing the broad implications of our research. We are grateful for your insight and have taken measures to ensure that our manuscript accurately reflects the current state of our research and its potential for future applications.

Technical Comments:

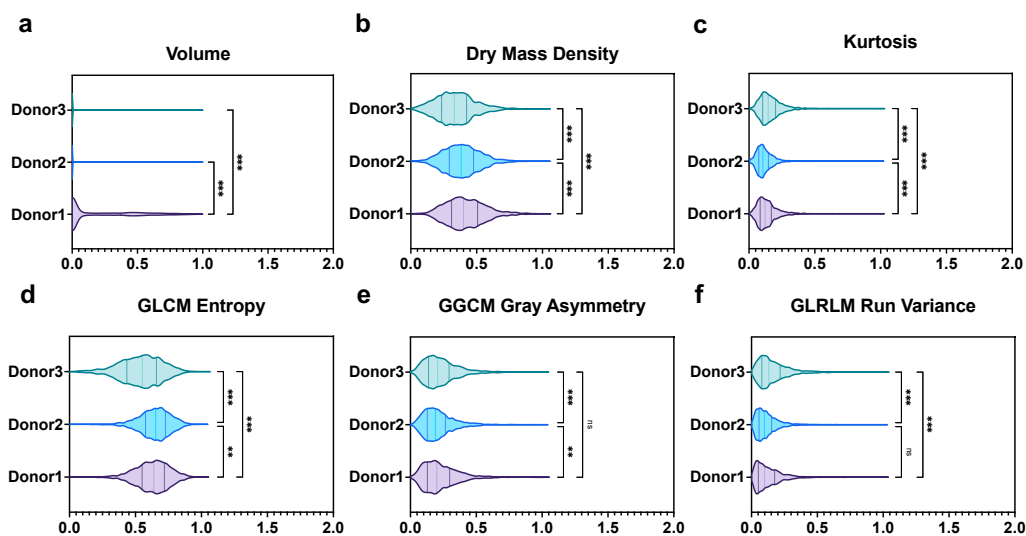
1. How did the authors address potential batch effect issues, common in imaging cytometry experiments? This concern is well-documented, for example:

* *RxRx1: A Dataset for Evaluating Experimental Batch Correction Methods*, DOI: 10.48550/arXiv.2301.05768

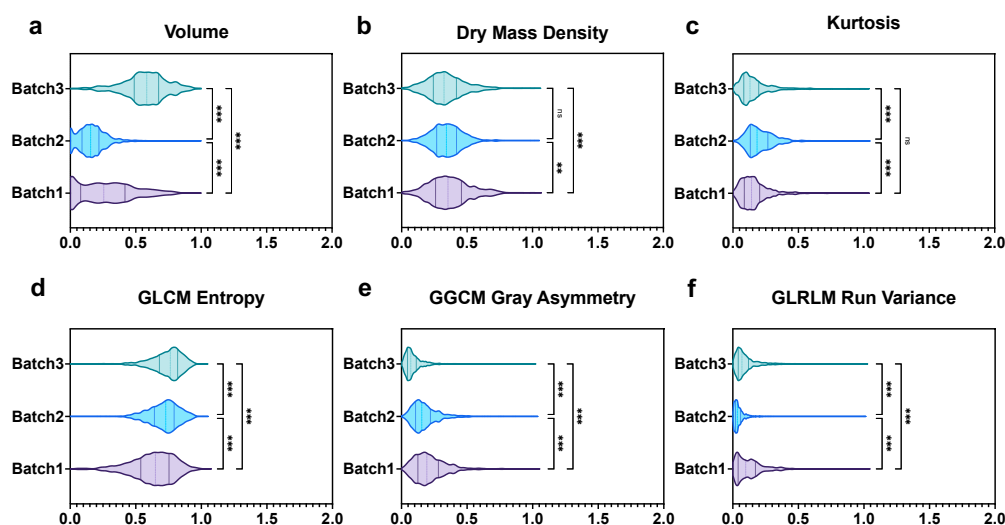
* *Information-Distilled Generative Label-Free Morphological Profiling Encodes Cellular Heterogeneity*, DOI: 10.1002/adv.202307591

Response: We appreciate your reference to the two papers that address this well-documented concern regarding the potential batch effect. We have carefully reviewed the literatures you provided and have taken steps to address and mitigate batch effects in our study.

In real clinical applications, blood test samples from new individuals will be encountered by our model, potentially introducing variances in morphological or textual features. To assess these variances, we used the Peripheral Blood Mononuclear Cells (PBMCs) from three different donors, and three batches of MDA-MB-231 cells. We plotted feature distributions for each donor/batch and observed distribution differences for certain cell types (see newly added Supplementary Fig. 19 and 20).

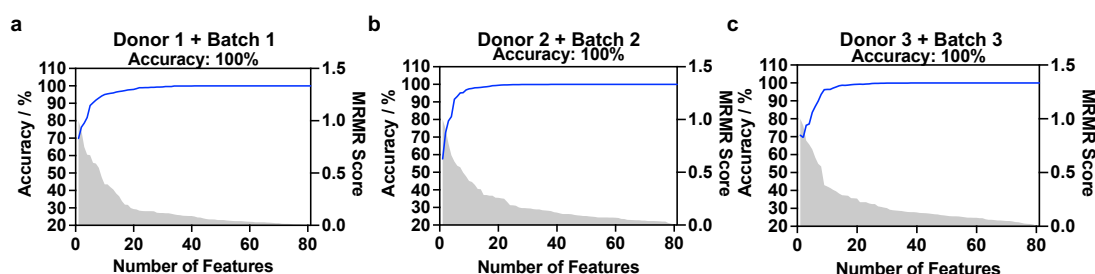


Supplementary Fig. 19 Violin plots of 6 QPM phase features of PBMC (N = 1,638 for Donor 1, N = 1,712 for Donor 2, N = 1,787 for Donor 3), including (a) volume (Bulk), (b) dry mass density (Bulk), (c) Kurtosis (First-order histogram), (d) entropy (GLCM), (e) gray asymmetry (GGCM), and (f) run variance (GLRLM). * indicates p-value < 0.001, ** indicates 0.001 < p-value < 0.01, ns indicates p-value > 0.05, based on ordinary one-way ANOVA pairwise comparisons (Tukey's test).**



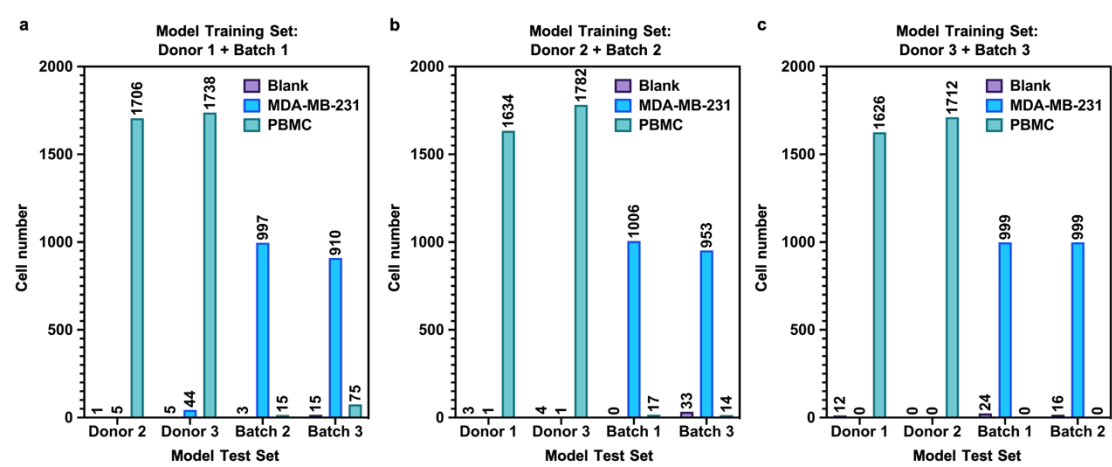
Supplementary Fig. 20 Violin plots of 6 QPM phase features of MDA-MB-231 (N = 1,023 for Batch 1, N = 1,015 for Batch 2, N = 1,000 for Batch 3), including (a) volume (Bulk), (b) dry mass density (Bulk), (c) Kurtosis (First-order histogram), (d) entropy (GLCM), (e) gray asymmetry (GGCM), and (f) run variance (GLRLM). * indicates p-value < 0.001, ** indicates 0.001 < p-value < 0.01, ns indicates p-value > 0.05, based on ordinary one-way ANOVA pairwise comparisons (Tukey's test).**

Following the approach outlined in relevant literature^{7, 8}, we explored the impact of these differences on the generalization of our model to new donors. Each time, one donor's PBMC sample was used for training the LDA model based on the MRMR-IFS strategy as mentioned before (see newly added Supplementary Fig. 21), while samples from the remaining donors were used for testing. This experiment was repeated by rotating the testing donor. The same approach was applied to MDA-MB-231 batches.



Supplementary Fig. 21 Incremental feature selection (IFS) curves for LDA machine learning algorithms, with the highest predictive accuracy of each model labeled accordingly. The gray shadow represents the MRMR scores of features. The blue curve illustrates the training accuracy rates.

Our results (see newly added Supplementary Fig. 22 and Table 13) demonstrated high accuracy rates in cross-validation, with discrimination accuracies above 0.90. For instance, modeling with Donor 1's PBMC data and Batch 1's MDA-MB-231 data achieved 99.6% and 98.2% discrimination accuracies for Donor 2 and Donor 3's PBMCs, and 91.0% and 98.2% for Batch 2 and Batch 3's MDA-MB-231 cells, respectively. All above results can be found in our revised Supplementary Information (see newly added Supplementary Note 8). These high cross-validation accuracies suggest that our model, trained with our MRMR-IFS strategy, has good generalization and universal applicability, showing potential for clinical sample detection.



Supplementary Fig. 22 Cross-validation results with LDA models. Models are trained on (a) Donor 1's PBMC with Batch 1's MDA-MB-231, (b) Donor 2's PBMC with Batch 2's MDA-MB-231, and (c) Donor 3's PBMC with Batch 3's MDA-MB-231, with remaining batches and donors serving as the test set in a rotating fashion. The bars represent the predicted outcomes for different cell types within the test set, including Blank (purple bars), MDA-MB-231 (blue bars), and PBMC (green bars).

Supplementary Table 13. Cross-validation results with LDA models

Training Dataset			Test Dataset			Test Dataset		
			PBMC			MDA-MB-231		
Blank	PBMC	MDA-MB-231	Donor 1	Donor 2	Donor 3	Batch 1	Batch 2	Batch 3
Blank	Donor 1	Batch 1	NA	99.6%	97.3%	NA	98.2%	91.0%
Blank	Donor 2	Batch 2	99.8%	NA	99.7%	98.3%	NA	95.3%
Blank	Donor 3	Batch 3	99.3%	100.0%	NA	97.7%	98.4%	NA

We attribute these high cross-validation accuracies to several key factors: 1) Fine-grained feature extraction: The high accuracy of our imaging system ensures that the extracted features are detailed and precisely represent cell features, which is crucial for the model's ability to distinguish between different cell types and states. 2) MRMR-IFS learning strategy: The use of MRMR score ranking for feature selection ensures that the most relevant and non-redundant features are chosen for model training. By incrementally adding new data to the model, the learning process can adapt to new information without forgetting previously learned patterns, thus improving the model's ability to generalize to new, unseen data. 3) Substantial number of cells per type: This ensures that the model is trained on a diverse set of samples, which helps in capturing the variability within each cell type.

However, considering that our current sample environment is relatively simple, we acknowledge that more complex classification problems and cell populations may require advanced modeling techniques. The two papers you provided have offered valuable insights into batch effect mitigation strategies. The paper titled "RxRx1: A Dataset for Evaluating Experimental Batch Correction Methods" introduces the RxRx1 dataset, designed to evaluate batch correction methods. It employs various deep learning techniques, including adaptive batch normalization (AdaBN) and gradient reversal, to remove batch information from image embeddings. The results show that AdaBN is particularly effective in generalizing to unseen batches. The other paper presents CytoMAD, a self-supervised multi-task learning strategy that distills biologically relevant cellular morphological information from batch variations. CytoMAD's approach of morphology distillation, paired with deep-learning image-contrast translation, offers additional insights into label-free cell morphology and has proven effective in integrating analysis across multiple data batches. These papers have inspired us to consider more advanced methods for our future work, particularly as we encounter more complex biological samples and classification challenges. **We have incorporated a prospect for further research on this aspect into the Discussions section (see lines 440-445 of the revised manuscript) and have cited the two references you provided.**

In conclusion, we have taken significant steps to address batch effects in our study and have plans to incorporate advanced techniques in our future work. We believe our current approach, combined with the insights from the literature, positions us well for accurate and robust analysis

in clinical settings. We kindly invite you to take a look at the updates we've made. Thank you again for your guidance.

2. Is there any attempt to extract the 3D refractive index of the cells in this system?

Response: We thank you for the inquiry which prompts us to reflect on the focus of our research and the future potential of our platform. Our study is mainly centered on the utilization of single-cell quantitative phase information to provide meaningful insights into cellular properties. We recognize that while the 3D refractive index is a significant parameter in QPM imaging, our approach has been to harness the wealth of information available from phase shifts. These phase shifts, being the integral of the refractive index over the specimen's thickness, offer a fundamental yet rich dataset for cell classification and characterization, as demonstrated in many literatures^{2, 5, 6, 8}. For example, Tsia et al.⁸ have successfully used phase maps to identify cancer subtypes and rare cell populations without the need for explicit 3D refractive index data. Similarly, the work by Vanapalli et al.² presents a label-free, high-throughput method for identifying and enumerating tumor cells in blood samples using digital holographic microscopy (DHM). Their approach, which relies on phase features such as size, maximum intensity, and mean intensity, has proven effective in categorizing and identifying cells in mixed populations. However, we acknowledge the potential benefits of incorporating 3D refractive index measurements in future iterations of our system. We are inspired by the work of others who have successfully measured 3D refractive index distributions of samples using digital holographic tomography¹, and we will consider these advancements as we evolve our system. In summary, while we have not yet attempted to extract the 3D refractive index of cells within this system, we are confident that our focus on phase analysis has yielded valuable insights into cellular characteristics. Lastly, we would like to express our gratitude once again for your question.

Response to Reviewer #3

Thanks for your comments and approval, which are greatly appreciated.

The response to your comments is list below:

1. Figure 2 (g) and (f) the hologram is cluttered with speckles and spurious fringes. How much these type of noise sources could affect the accuracy for reconstructing the phase maps? The source of these noises and solving these problems can be found in the following article: "Quantitative Phase Microscopy and Tomography: Techniques using partially spatially coherent monochromatic light, IOP Publishing 2022."

Response: Thank you for your insightful comments on the noise observed in Figure 2(g) and (f) of our manuscript. We have taken your feedback seriously and have conducted a detailed analysis of the phase noise in our DHM system. We have included this part of the research in both the manuscript (see lines 120-127) and the supplementary information (see lines 228-267 of the revised Supplementary Note 2, and newly added Supplementary Figure 7), and have cited the reference you provided.

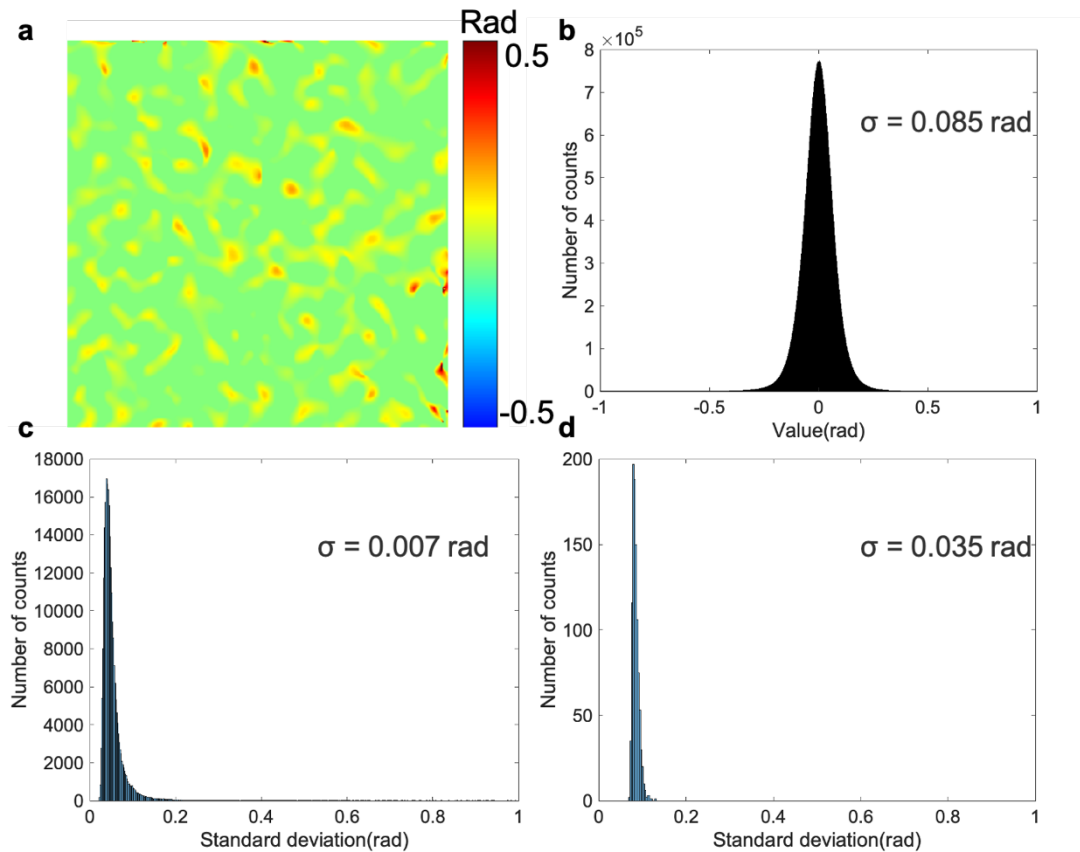
The referenced article from IOP Publishing 2022 has been instrumental in our understanding of temporal phase noise, which arises from path length differences between reference and object beams due to mechanical vibrations, air fluctuations, and electronic noise. Spatial phase noise, intrinsically linked to speckle, is due to spatial intensity fluctuations from interfering spurious fields at the detector. A laser's high spatiotemporal coherence leads to the occurrence of speckle noise and spurious fringes leading to poor spatial phase sensitivity and inhomogeneous illumination. All these factors significantly influence the phase measurement accuracy of QPM systems and lead to poor image quality.

To quantify the effect, our approach to measuring the spatiotemporal phase noise involved capturing a series of background holograms (496×496 pixels) at 10-second intervals over a period of approximately 2.8 hours, totaling 1,000 images. Using the first image of the sequence as a reference, we determined the phase fluctuation for each subsequent image. The newly added Supplementary Fig. 7a corresponds to the phase fluctuation in the first frame of this series. Supplementary Fig. 7b shows the histogram distribution of the overall spatiotemporal phase fluctuation which had a standard deviation of 0.085 rad. This value represents the overall spatiotemporal noise in our system.

We began by analyzing the spatial noise. [Supplementary Fig. 7c](#) shows the histogram distribution of spatial noise, with a mean of 0.085 rad and a standard deviation of 0.007 rad. Given that the average phase height of typical cells we measure is around 3-5 rad (as shown in [Supplementary Fig. 9c](#)), we believe this level of noise is significantly smaller than the cell's phase height, and thus, the impact of this noise on our measurements should be minimal. Next, we analyzed the temporal noise. [Supplementary Fig. 7d](#) illustrates the histogram distribution of temporal noise. The results show a mean of 0.053 rad, with a standard deviation of 0.035 rad. This is considerably lower than the 0.2 rad standard deviation reported for typical Mach-Zehnder configurations in DHM, demonstrating the high stability of our measurement system. In conclusion, our measurements indicate that our DHM system is capable of achieving high stability and sensitivity in phase distribution, with the noise levels being well below what would significantly affect our ability to accurately measure cell phase. [We have detailed the specific measurement formulas and experimental procedures in the revised Supplementary Information dedicated to DHM parameter determination \(see the revised Supplementary Note 2\).](#)

Looking ahead, we are committed to further optimizing our DHM setup to reduce noise even further. We are also exploring new QPM configurations, such as the use of partially coherent light, which may further reduce noise. These configurations, as discussed in the literature you provided, offer promising avenues for improvement. We are actively considering these advancements to further enhance the stability and performance of our platform.

Thank you again for your valuable input, which has significantly contributed to the quality of our research. We hope this response alleviates your concerns and demonstrates our system's capability to perform accurate cell phase measurements with minimal noise interference.



Supplementary Fig. 7 Noise measurement of our DHM. **a** Spatial noise distribution of background at first frame. **b** Spatiotemporal noise histogram of 1,000 frames in approximately 2.8 h time-series. **c** Histogram of the standard deviation of spatial noise. **d** Histogram of the standard deviation of temporal noise.

2. Figure 3 (a), healthy red blood cells are generally donut shape, whereas in this figure last column the shapes do not look like donut. What could be the reason?

Response: Thank you for your attentive observation regarding Figure 3 (a). The cell line used in this study, THP-1, is a human monocytic leukemia cell line, which belongs to the category of white blood cells. Upon reviewing the Figure 3 (a), we realized that the label "Blood" above THP-1 may have caused confusion, which was an oversight on our part. **We apologize for any misunderstanding this may have led to and have changed "Blood" to "Leukemia" to prevent future potential confusion (see the revised Figure 3).**

Additionally, we have included data and imaging for red blood cells in our research, where the characteristic donut-like shapes are clearly visible (see Figure R2, and newly added Figure 7a

of the revised manuscript), aligning with your expectations and the established knowledge on red blood cell morphology.

We have corrected the labeling in Figure 3 (a) and ensure that all figures and their descriptions accurately reflect the cells being presented. We are grateful for the opportunity to clarify this point and for your understanding as we make these necessary amendments.

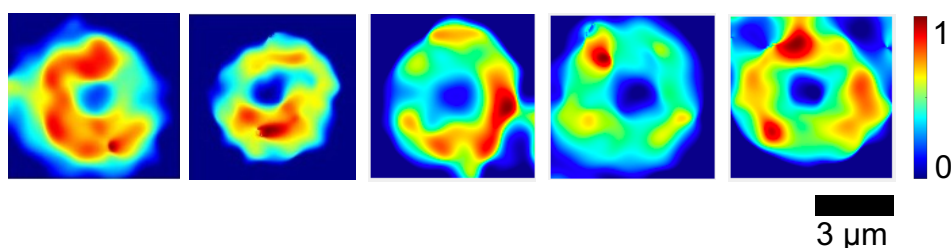


Figure R2. Single-cell QPM phase maps of human red blood cells. The color bar denotes the scale of normalized optical path difference (OPD) to reflect phase information (Scale bar: 3 μm).

3. The entire process includes QPM-IFC image recording, phase reconstruction procedure and machine learning algorithms for cell identification. Is it possible to make entire process real time?

Response: Thank you for your insightful question regarding the real-time capability of our process. Our SMHS platform is currently in the proof-of-concept phase, where we have prioritized innovative integration, precise control and accurate analysis over real-time processing. While our current platform does not make entire process real time, we believe that our platform has several favorable conditions that could facilitate real-time processing. For instance, our phase reconstruction and feature extraction algorithms are of low complexity, which do not involve computationally intensive processing such as digital refocusing or high-resolution 3D imaging. Our MRMR-IFS strategy optimizes machine learning model training by achieving high accuracy using the fewest features as possible, making it suited for real-time applications. Additionally, our entire workflow is standardized, including standardized DHM imaging conditions, microfluidic focusing conditions, and algorithmic procedures, which are conducive to achieving real-time and automated processing. Furthermore, our platform's measurement stability and compatibility with high-throughput computing resources provide a solid foundation for potential real-time capabilities.

Our review of the literature¹⁷⁻¹⁹, particularly the three papers provided, has provided us with valuable inspiration. The first paper¹⁷, "Quantitative phase imaging flow cytometry for ultra-large-scale single-cell biophysical phenotyping," demonstrates the feasibility of high-throughput imaging and analysis, which inspires us to consider how we might scale our system to handle larger datasets in real-time. The second paper, "Machine learning based real-time image-guided cell sorting and classification,"¹⁸ shows how machine learning can be integrated into a real-time workflow, suggesting that our own machine learning models could be optimized for real-time decision-making. Lastly, "Intelligent image-activated cell sorting"¹⁹ presents a system that uses FPGA technology to achieve rapid cell sorting, which could be adapted to our phase reconstruction and feature extraction processes to achieve real-time performance. These studies suggest that with further optimization and technological advancements, real-time operation is indeed a promising goal.

Your question has not only highlighted a critical area for future exploration but has also directed us to include a discussion on this topic within the manuscript (see lines 445-448). We appreciate the opportunity to refine our approach based on your feedback.

4. Quantitative phase microscopy of moving cells and microparticles have been done earlier using waveguide trapping and propulsion “Quantitative phase microscopy of red blood cells during planar trapping and propulsion,” *Lab on a Chip* 18 (19), 3025-3036 (2018). How do you compare your technique with these techniques.

Response: Thank you for your question regarding the comparison between our technique and the one presented by Ahluwalia et al. in their 2018 publication in *Lab on a Chip*. Below, we outline the key distinctions between our technique and theirs:

1) QPM architecture

Ahluwalia et al. employed a reflection-type QPM setup based on a Linnik interferometer, capable of capturing off-axis holograms and rapidly recovering phase information via Fourier transformation. Our study introduced a transmission-type QPM based on a Mach-Zehnder interferometer, specifically off-axis image plane DHM. The Mach-Zehnder interferometer is known for its superior interference contrast and optical path stability, essential for accurate analysis. Therefore, we chose QPM technology based on this interferometer.

2) Cell manipulation techniques

Ahluwalia et al. utilized planar waveguide trapping (WT) to propel cells and simulate their flow in micro-vessels, focusing on studying red blood cell (RBC) deformability. Our technique employed a spatial hydrodynamic focusing microfluidic module, which not only allows precise focusing of cells and particles of varying properties and sizes in both vertical and horizontal directions but also provides strict spatial confinement, eliminating issues such as cell floating and overlap that can compromise single-cell identification accuracy.

The differences in cell manipulation techniques most directly lead to the difference in defocus management. We can see that in their study, numerical refocusing was necessary due to dynamic defocusing issues that can alter the phase of RBCs during manipulation. "A previously proposed focus correction algorithm based on the angular spectrum propagation approach was employed in post-processing for an accurate assessment of RBC phase maps, thus removing the defocusing issue." Unlike the method where dynamic defocusing is a concern, our approach ensures that cells remain in focus throughout the process. Our integrated innovation allowed us to capture in-focus holograms of single cells and particles directly, eliminating the need for digital refocusing or additional compensation, which is time-consuming.

3) Types of features extracted

Ahluwalia et al.'s study was centered on morphological parameters of RBCs, such as surface area, volume, and sphericity, without delving into higher-order texture features. However, our research established a hierarchical QPM feature framework including 14 bulk features, 17 first-order histogram features, and 50 high-order texture features, offering a comprehensive characterization of single-cell phenotypes, which formed the foundation for our classification of large-scale heterogeneous cell populations.

4) Analysis methods

Ahluwalia et al. analyzed RBCs deformation by comparing morphological parameter changes before and after capture. We went beyond feature comparison by implementing an incremental feature selection strategy based on the Maximun-Relevance and Minimun-Redundancy (MRMR) algorithm, which was integrated into various classic machine learning models, significantly enhancing classification accuracy and efficiency.

In summary, our technique showcases innovations and improvements over existing methods in terms of QPM architecture, cell manipulation techniques, feature extraction types, and analysis

methods. We have made progress, especially in integrated innovation, precise system control, and the analysis of heterogeneous cell populations. We have cited Ahluwalia et al.'s work in our manuscript and have revised the Discussions section to reflect the distinctions and advantages of our technology (see lines 394-429). We appreciate your valuable comments and suggestions and look forward to your further guidance and feedback.

5. In figure 2 (a) and (b) although it is quite clear that focusing of moving particle can be achieved, nevertheless how do you calibrate that the focusing is perfect ~100%.

Response: We appreciate your insightful question regarding the calibration of focusing in Figures 2(a) and (b). It is important to clarify that achieving perfect focusing of each individual moving particle at 100% is an ideal scenario, yet it is not always practical in experimental settings. The primary purpose of these figures is to visually demonstrate the process of spatial hydrodynamic focusing, rather than to prove perfection. However, we fully understand the importance of ensuring the accuracy and reliability of our experimental setup, and we have implemented several calibration steps:

First, we calibrate our system based on the relationship of flow velocity ratios. We determine the optimal flow velocity ratios based on our theoretical simulation and experimental validation results (see Supplementary Table 1 and 2), which correlate the velocity of sheath and sample flows to the width and height of the focused particle stream. Given that the standardized size of our microparticles is known, we adjust the flow rates accordingly to achieve the desired focusing conditions.

Second, we make pre-experiment focusing adjustments. Prior to actual measurements, we perform a brief pre-sampling trial to fine-tune the XYZ-axis knobs of the stage for placing microfluidic chips. This process begins with loading a small volume of microparticle suspension into the microfluidic channel. We then proceed to adjust the XYZ-stage to optimize focus, observing the microparticles under our imaging system and making incremental adjustments to the XYZ-axis knobs until the microparticles are in sharp focus, as indicated by the clarity and definition of their holograms. This iterative process may include adjusting the Z-axis to control the axial position, the X- or Y-axes to control the lateral position, and sometimes even minor adjustments to the angle of illumination to achieve optimal holographic contrast. This pre-experiment calibration is essential for ensuring that the subsequent data

collection and analysis are based on high-resolution, in-focus holograms, which are crucial for accurate phase reconstruction and subsequent analysis.

Third, we validate the focusing efficiency statistically. Although achieving perfect focusing for each individual moving microparticle presents a challenge, we rely on statistical analysis of the group data to demonstrate the precision of our technique. The consistency and reproducibility of our results (see Figure 2h-k) across a large number of particles indicate that our focusing approach is highly effective.

We hope this explanation clarifies how we ensure the focusing efficiency of our system. We are confident that our approach, while not guaranteeing 100% perfection for each particle, achieves a high level of accuracy and consistency across the population of particles, which is crucial for our analysis of heterogeneous cell populations.

We appreciate your scrutiny and are committed to addressing any concerns that may arise to ensure the integrity and robustness of our research.

6. The experimental set-up for QPM is extremely sensitive to external vibrations leading to erroneous phase measurement. How much this configuration will affect phase measurement in the present work? A possible solution for this off-axis configuration is on-axis QPM “Highly stable wide-field common path digital holographic microscope based on a Fresnel biprism interferometer, OSA Continuum 1 (1), 48-55 (2018).

Response: Thank you for your concern regarding the sensitivity of our QPM experimental setup to external vibrations and its potential impact on phase measurement. We also appreciate your reference to the on-axis QPM setup as a potential solution to mitigate such issues.

We have carefully reviewed the paper you provided, which also focuses on the temporal stability as an indicator of performance. We used cage-like structure design which can enhance system stability by dampening external vibrations and maintaining precise alignment of optical components. This design contributed to the excellent spatiotemporal stability of phase measurement (see lines 228-267 of the revised Supplementary Note 2, and newly added Supplementary Figure 7). Specifically, the standard deviation of temporal phase noise (0.035 rad) is significantly lower than that of typical Mach-Zehnder configurations in DHM (0.2 rad), highlighting the superiority of our system. The standard deviation of spatial phase noise (0.007

rad) is much lower than the average phase height of typical cells (3-5 rad), thus ensuring minimal impact on measurements.

We are grateful for the literature you have provided, which has broadened our perspective on DHM optical path design. We have also discussed in detail the prospects for enhancing the performance of each module in the future (see lines 449-450 of the revised manuscript) and have cited the paper you provided. As we move into the optimization phase of our work, we will certainly consider further improvements to our optical setup. However, at this stage, our focus is on validating the concept of our entire platform, and thus, we have not yet undertaken extensive work in this area.

We hope this response addresses your concerns and demonstrates our commitment to ensuring the accuracy and reliability of our phase measurements. We are confident that our current setup, combined with the potential for future improvements, will provide robust results for our cellular analysis studies.

7. How fast is the proposed system for recording and reconstruction of phase maps of live cells of irregular shapes and moving randomly? Can the present system trace nanoscale motion of the fast-moving samples like the results presented in a recent study” Characterizing the consistency of motion of spermatozoa through nanoscale motion tracing, Fertility and Sterility (2024).

Response: Firstly, we would like to address your inquiry regarding the speed of our system in recording and reconstructing phase maps of live cells.

In terms of recording speed, our microfluidic module is estimated to have a throughput of around 1,600 particles per second, as mentioned in our Discussions (please refer to lines 430-435). This figure is close to the level of commercial imaging flow cytometry (IFC) systems. However, the current detection throughput is primarily limited by the camera's frame rate of only 30 frames per second (fps). This means that, for the time being, we can only operate at a lower flow rate. We are transparent about this constraint in our manuscript (see lines 435-438) and acknowledge that our system's detection throughput is lower compared to traditional commercial IFC systems and some state-of-the-art quantitative phase imaging (QPI) cytometers. We recognize that our platform is still in the early stages of proof-of-concept. While detection throughput is a significant consideration, it is not the primary focus at this stage of development.

We are fully aware that for practical applications, the throughput will need to be enhanced. We plan to address this by modifying the system configuration, including the use of cameras with faster frame rates (in the thousands to millions) to increase the throughput in future iterations. Unfortunately, due to time and financial constraints, we have not been able to complete the camera upgrade, and we have refined the limitations in our Discussions.

In terms of phase reconstruction speed, as mentioned in our revised manuscript and Supplementary Information (see lines 133-137 of the revised manuscript, lines 330-354 of the revised Supplementary Note 3, and newly added Supplementary Table 5), the reconstruction of a single-cell hologram (496×496 pixels) is completed within about 0.34 seconds using lab-written MATLAB codes on our current laptop equipped with a 2.8 GHz Intel Core i7 CPU and 16 GB of RAM. Here, we have listed the duration of each phase reconstruction step (see newly added Supplementary Table 5), which is the average time obtained by measuring the reconstruction time of 20 single cell holograms. We would like to emphasize that this reconstruction time is potentially advantageous compared to the similar study. In their traditional holographic reconstruction process, the phase reconstruction of a single-cell hologram (384×384 pixels) took 7.71 seconds (their computer configuration was a 64Gb RAM Intel® Core™ i9-9900K CPU). It is evident that numerical refocusing is the most time-consuming step, and our study's innovative integration help us to omit this step, thereby gaining a significant advantage in the reconstruction time of phase maps.

Supplementary Table 5. Comparison of QPM phase reconstruction workflow and speed

QPM phase reconstruction workflow in our research					
Frequency filtering	Numerical reconstruction	Phase extraction	Phase unwrapping	Phase correction	Total
0.084 s	0.009 s	0.004 s	0.056 s	0.189 s	0.343 s
QPM phase reconstruction workflow in other research ^{14*}					
Holographic demodulation	Numerical refocusing	Phase extraction	Aberration compensation	Denoising & Unwrapping	Total
0.06 s	4.46 s	0.01 s	0.42 s	2.76 s	7.71 s

*Supplementary Reference 14. Pirone, D. et al. Speeding up reconstruction of 3D tomograms in holographic flow cytometry via deep learning. *Lab. Chip* **22**, 793-804 (2022).

Subsequently, we would like to address your second question regarding the capability of our system to trace nanoscale motion of fast-moving samples. Upon careful consideration, we must

clarify that the current configuration of our platform is not specifically designed to capture nanoscale motion at the speeds and sensitivities required for tracing the high-precision movements as demonstrated in the study you mentioned. We have identified several key factors that contribute to this limitation: 1) Resolution: The current resolution of our system is limited to approximately 0.7-0.8 μm (see Supplementary Note 2). While this resolution is sufficient for many applications, it falls short of the nanoscale resolution required to accurately track the fine details of fast-moving particles at the level demonstrated in the referenced study. 2) Measurement mode: Our system is designed to operate in an imaging flow cytometry (IFC) manner, where particles are focused and constrained to move through a central region. This design is well-suited for high-throughput analysis of single-cell quantitative phase microscopy (QPM) but may not be ideal for capturing the irregular and complex motion patterns often exhibited by fast-moving samples such as sperm cells. 3) Camera frame rate: The camera used in our current setup has a frame rate of 30 fps. This frame rate may not be sufficient to capture the rapid movements of fast-moving samples with the necessary precision. We would like to clarify that our system excels in providing high-fidelity single-cell QPM imaging and reconstruction without digital refocusing, which reduces computational time significantly and allows for high-throughput analysis.

We are actively exploring upgrades to our platform, including the use of high-speed cameras and advanced image processing algorithms, which could potentially enhance our system's capabilities in the future. We believe that continuous improvement and adaptation to new challenges are crucial for the development of cutting-edge scientific tools.

We are grateful for your feedback, as it highlights areas where our technology can be further developed. **We have also cited this literature as a potential application expansion for the future (see lines 440-445 of the revised manuscript).** We are dedicated to pushing the boundaries of our SMHS platform's performance and will consider the insights from your question in our future research and development efforts.

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Point-by-point Response to Reviewers' Comments

We sincerely appreciate all the Reviewers for their continued evaluation and invaluable comments. Below we submit a point-by-point response letter in which we have provided detailed responses to each comment from the Reviewers and made corresponding revisions in our revised manuscript.

Response to Reviewer #1:

Thanks for your expertise and supportive evaluation, guiding us to improve our manuscript.

The response to your comments is list below:

The answers of the authors to my previous remarks made my option stronger that the novelty of the paper is unsuitable for Nature Communications. 30fps is not attractive for cell analysis. Much higher throughputs have already been demonstrated by several groups. Hydrodynamic focusing is not the critical issue. I therefore think that the paper is more suitable to biomedical optics or cytometry journals.

Response: We sincerely thank you for the time you have invested in evaluating our manuscript. Your comments have provided us with valuable insights, and we appreciate the opportunity to address your concerns regarding the novelty and suitability of our work for *Nature Communications*.

1) Why our work is suitable for *Nature Communications*

We appreciate your suggestion. However, we firmly believe that our study aligns well with the growing trend of interdisciplinary innovation encouraged by *Nature Communications*. As stated on the journal's "Aims & Scope" page, *Nature Communications* is dedicated to publishing high-quality research across various scientific disciplines, emphasizing significant advances that are of interest to specialists within each field. Our research integrates microfluidics, digital holography, feature engineering and machine learning to address critical challenges in cancer heterogeneity analysis. This interdisciplinary approach not only pushes the boundaries of single-cell quantitative phase microscopy (QPM) but also has significant potential for practical application. Therefore, we believe that our work fits well within the cross-

disciplinary framework emphasized by *Nature Communications* and represents an important advance in the field.

2) Addressing the concerns regarding 30 fps

We acknowledge that the imaging rate of 30 fps may seem limited compared to other studies. However, we would like to emphasize that our system design focuses on integrated innovation rather than maximizing throughput through hardware advancements. Actually, as we noted in our previous revision (see lines 444-447 of the revised manuscript), our microfluidic module alone has achieved a peak throughput of approximately 1,600 particles/s (at a sample flow rate of 10 $\mu\text{L}/\text{min}$ and a particle concentration of $\sim 1 \times 10^7$ particles/mL), which is comparable to commercial imaging flow cytometry (IFC) systems at 2,000 particles/s.

Moreover, our system achieves a 22.7-fold improvement in single-cell phase reconstruction time, reducing it from 7.71 s to 0.34 s. This significant enhancement in efficiency addresses a critical bottleneck in the field of QPM-IFC. The dramatic reduction in phase reconstruction time is equally advantageous and attractive for cell analysis. We believe that this innovation, which emphasizes holistic system-level improvements rather than focusing on a single parameter, represents a valuable contribution to the field. It highlights a shift from a "parameter competition" to a "solution-oriented innovation", which we believe is more aligned with the journal's emphasis on interdisciplinary advancements.

3) Addressing the concerns regarding hydrodynamic focusing

We would like to re-emphasize the importance of spatial focusing for our QPM imaging and research objectives. Upon reflection, we recognize that our original explanation was insufficient, particularly in highlighting the full scope of spatial focusing's advantages over planar focusing, which led to the ambiguity in understanding the necessity of spatial focusing. We have now thoroughly revised and expanded our discussion to address this issue comprehensively.

Actually, spatial focusing serves three critical functions. First, it significantly enhances the accuracy and efficiency of amplitude and phase reconstruction. Second, it strictly confines the single-cell flow to the center of the microchannel, which is essential for optimizing the automated/standardized acquisition of single-cell holograms. Third, it improves the consistency of amplitude and phase measurements by reducing variability in the reconstructed data.

i. Enhancing reconstruction accuracy and efficiency

As shown in Fig. 2d and e, in the initial state, the sample flows naturally into the microchannel without any sheath flow constraints. Particles are horizontally dispersed and vertically stacked. Moving to the intermediate state, planar focusing is achieved when the sample flow is first constrained by vertical sheath flows, aligning the cells into the same vertical plane. As shown in Fig. 2h-k, this step significantly improves the accuracy of amplitude and phase reconstruction compared to the initial state, as the reconstruction precision is largely determined by the enhancement in axial (Z-axis) localization. This addresses a key issue in previous QPM-IFC studies based on hydrodynamic focusing, as described in lines 67-72 of the revised manuscript:

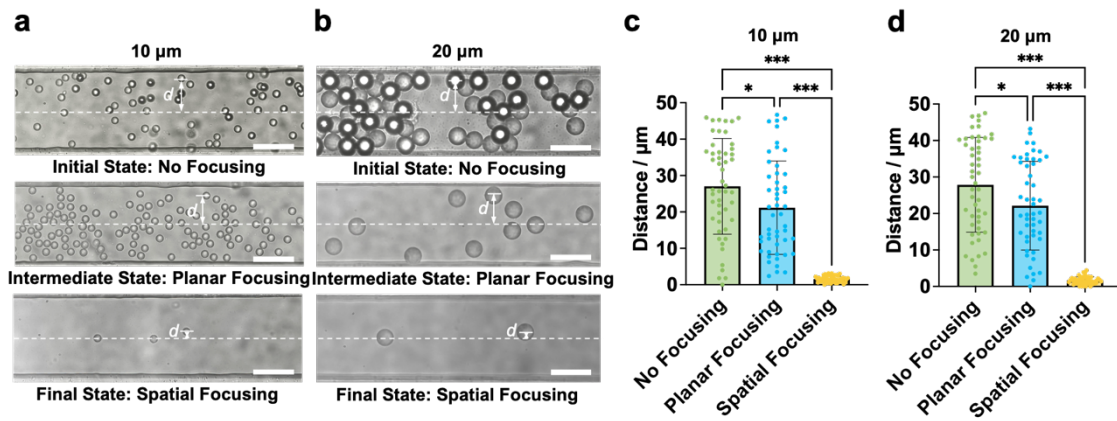
"Axially, cells are either unconstrained or loosely confined, leading to cell dispersion and potential overlap which can compromise single cell identification precision. While digital refocusing algorithms can help accurately locate cells at different depths, they are computationally expensive and time-consuming, slowing down IFC's rapid workflow. The lack of axial confinement also causes cell oscillation, imaging artifacts and blurriness, and finally loss of key textures in QPM measurements."

Therefore, our approach significantly improves this aspect, highlighting the enhanced effectiveness and novelty of our method compared to existing studies. Finally, our system achieves a reconstruction time of approximately 0.34 seconds per unit, accounting for only 4.41% of the typical process (~7.71 seconds)¹.

ii. Optimizing automated single-cell hologram acquisition

The final state, spatial focusing, is achieved with further constraining the sample flow in the horizontal direction. This step accomplishes the second goal of strictly localizing the single-cell flow to the center of the microchannel. As illustrated in Fig. 2d and e, planar focusing still leaves particles dispersed distributed within the plane. To extract information from individual particles, extensive particle localization and image segmentation are required over a larger field-of-view, which will increase computational complexity and introduce potential errors. In contrast, under spatial focusing, particles are concentrated in a streamlined region at the channel center, enabling direct capture of single-particle images within a fixed central acquisition window (496 pixels $\times$ 496 pixels, 31 μm $\times$ 31 μm).

We have now supplemented our analysis with quantitative data on spatial localization by measuring the average distance (d) of the particles' centers from the microchannel centerline. As shown in Supplementary Fig. 11 and Table 6 of the revised Supplementary Information, compared with planar focusing, we find that spatial focusing significantly reduces this distance and tightens the distribution. Specifically, for 10 μm and 20 μm microparticles, the average distances of their centers from the channel centerline are $(1.5\pm0.8) \mu\text{m}$ and $(1.6\pm1.0) \mu\text{m}$, respectively. The highly efficient spatial focusing not only proves spatial localization precision but also provides a stable and efficient foundation for automated single-cell image acquisition. Additionally, because we can directly capture single-cell holograms at fixed position, localization and segmentation algorithms are unnecessary, further streamlining our phase reconstruction process.



Supplementary Fig. 11 Distance distribution of microparticles under different focusing states. (a) and (b) show the observation of 10 μm (a) and 20 μm (b) polystyrene microspheres in microfluidic channels under difference focusing conditions in white light illumination. d measures the distance of single particle centers from the microchannel centerline. (c) and (d) show the statistical results of the distance under different focusing conditions ($N=50$).

Supplementary Table 6. Distance distribution of microparticles under different focusing states

Focusing State	No Focusing	Planar Focusing	Spatial Focusing
10 μm microparticles	$(27.1\pm13.1) \mu\text{m}$	$(21.2\pm12.8) \mu\text{m}$	$(1.5\pm0.8) \mu\text{m}$
20 μm microparticles	$(27.9\pm13.0) \mu\text{m}$	$(22.1\pm12.1) \mu\text{m}$	$(1.6\pm1.0) \mu\text{m}$

iii. Improving measurement consistency

Furthermore, spatial focusing also improves the consistency of amplitude and phase measurements. As illustrated in Fig. 2h-k, while planar focusing improves accuracy, it still results in higher variability in measurement values. Spatial focusing, through its dual constraints in both vertical and horizontal directions, reduces this variability. We have introduced the Coefficient of Variation (CV), the ratio of the standard deviation to the mean, as an important metric for comprehensively evaluating the precision (accuracy) and stability (consistency) of a measurement system in IFC. Our calculations (see Supplementary Table 7) indicated that the CVs for amplitude and phase measurements reached 3.3%/4.3% (10 μm microspheres) and 1.9%/4.7% (20 μm microspheres) under spatial focusing. These values align with previous studies on the measurement consistency in flow cytometry where CVs are typically $<5\%^2$, demonstrating the effectiveness of spatial focusing in achieving reliable and reproducible single-cell analysis. Thus, the quantification of CV values further highlights the necessity of spatial focusing.

In conclusion, we have added more detailed explanations and supporting data to better illustrate these points (see lines 357-405 of the revised Supplementary Information, Supplementary Fig. 11, Table 6 and 7). We have also revised our manuscript to more accurately reflect the necessity of spatial focusing. The specific revisions are as follows:

lines 208-233: Through quantitative comparisons, spatial focusing demonstrates three critical advantages over planar/no focusing. First, it enhances the accuracy and efficiency of amplitude and phase reconstruction. The amplitude-based particle sizes were derived from the mean of measured length/width in reconstructed amplitude maps, while phase-based sizes were calculated via equation (1) using peak-to-valley phase differences (see Methods section). Statistical analysis of 50 microspheres per condition confirmed spatial focusing's necessity for both amplitude (Fig. 2h, j) and phase (Fig. 2i, k) reconstruction. For instance, the phase size values of 10 μm and 20 μm microspheres without focusing were 7.13 μm (Fig. 2i) and 16.5 μm (Fig. 2k), respectively. However, with spatial focusing, these values were refined to 10.4 μm (Fig. 2i) and 19.8 μm (Fig. 2k), closely aligning with their true sizes. This accuracy improvement, coupled with a reduction in reconstruction time (0.34 s vs. 7.71 s per hologram) through eliminated digital refocusing needs (Supplementary Table 5), establishes spatial focusing as a precision-efficiency approach. Second, the strict

spatial localization confined particles to a narrow central stream with sub-2 μm lateral deviation (see Supplementary Fig. 11a-d and Table 6 for details), enabling direct imaging through a fixed acquisition window. This approach eliminates the need for particle tracking and segmentation algorithms typically required in planar or non-focusing processes, which can introduce computational complexity and potential misidentification errors, thus laying the foundation for automated hologram capture. Finally, spatial focusing enhanced measurement consistency through flow stabilization. By constraining the particles' trajectory both vertically and laterally, the numerical distribution of amplitude and phase of the particles displayed more compact (Fig. 2h-k). The coefficient of variation (CV), a metric indicative of the precision and stability of hydrodynamic focusing and measurement systems in IFC, was employed to evaluate the platform comprehensively. Our calculations (see Supplementary Table 7) indicated that the CVs for amplitude and phase measurements reached 3.3%/4.3% (10 μm microspheres) and 1.9%/4.7% (20 μm microspheres) under spatial focusing. These values align with previous studies on the measurement consistency in flow cytometry where CVs are typically $< 5\%$ ⁶⁶.

4) Emphasizing interdisciplinary innovation and superior performance

Finally, we would like to reiterate that our work is not merely a combination of existing technologies but represents a significant interdisciplinary innovation that addresses critical challenges in single-cell analysis. Our platform achieves several key advancements:

- i. **Superior spatiotemporal noise stability of DHM:** Our cage-structured DHM demonstrates exceptional spatial and temporal stability, with a spatial noise deviation of 0.035 rad, significantly lower than the traditional Mach-Zehnder DHM configuration (0.2 rad), and a temporal noise deviation of 0.007 rad, which is negligible compared to the cell phase height (3-5 rad).
- ii. **Efficient single-cell phase reconstruction:** Thanks to our integrated innovations, our platform achieves precise quantitative phase reconstruction without the need for digital refocusing algorithms. The single-cell hologram reconstruction time is only 0.34 s, representing just 4.41% of the traditional process (approximately 7.71 s).
- iii. **High measurement precision and consistency:** Our platform achieves high accuracy and consistency in QPM-IFC. The amplitude measurement CV values for

10 μm and 20 μm particles are 3.3% and 1.9%, respectively, while the phase measurement CV values are 4.3% and 4.7%, respectively. These values align with previous studies on the measurement consistency in flow cytometry where CVs are typically $<5\%$.

- iv. **Broad applicability and effectiveness:** Our study validates the focusing performance across different particle sizes (10 and 20 μm) and various cell types, including five types of cancer cells (MDA-MB-231, A549, HCT116, HeLa, THP-1), breast cancer-related cells (MCF-7, T47D, BT474, MDA-MB-361, SK-BR-3, HCC1937, MDA-MB-231, MCF-10A), and blood cells (human RBCs and PBMCs). This extensive validation demonstrates the broad applicability and versatility of our system, which features the most diverse range of cell types among current studies.

In summary, we believe that our work not only meets the high standards of *Nature Communications* in terms of interdisciplinary innovation and significance but also demonstrates superior performance and broad applicability. We have revised our manuscript to better highlight these points and to provide a clearer picture of the potential impact of our work. Thank you once again for your valuable feedback.

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Response to Reviewer #2:

Thanks for your expertise and supportive evaluation, guiding us to improve our manuscript.

The response to your comments is list below:

I have read the revised manuscript by Jiayi Shi et al. In general, the authors have addressed most of my comments/concerns, including acknowledging some of the limitations. I think there are a few key points that should be further clarified and revised:

1. In terms of cell/particle focusing, I still find it difficult to understand what's the novelty of the so-called "spatial focusing". The authors claimed that "The findings revealed that spatial focusing is essential for the accurate reconstruction of both amplitude (Fig. 2h, j) and phase (Fig. 2i, k)." But actually, the planar focusing, which is simpler in implementation, can achieve similar accuracy in both amplitude and phase (also seen from Fig. 2h-k). So, I wonder why we need spatial focusing in this case if the planar focusing method already yields the similar performance.

Response: Thank you very much for raising this important question regarding the novelty and necessity of spatial focusing in our study. Upon reflection, we recognize that our original explanation was insufficient, particularly in highlighting the full scope of spatial focusing's advantages over planar focusing, which led to the ambiguity in understanding the necessity of spatial focusing. We have now thoroughly revised and expanded our discussion to address this issue comprehensively.

Actually, spatial focusing serves three critical functions. First, it significantly enhances the accuracy and efficiency of amplitude and phase reconstruction. Second, it strictly confines the single-cell flow to the center of the microchannel, which is essential for optimizing the automated/standardized acquisition of single-cell holograms. Third, it improves the consistency of amplitude and phase measurements by reducing variability in the reconstructed data.

i. Enhancing reconstruction accuracy and efficiency

As shown in Fig. 2d and e, in the initial state, the sample flows naturally into the microchannel without any sheath flow constraints. Particles are horizontally dispersed and vertically stacked. Moving to the intermediate state, planar focusing is achieved when the sample flow is first constrained by vertical sheath flows, aligning the cells into the same vertical plane. As shown in Fig. 2h-k, this step significantly improves the accuracy of amplitude and phase reconstruction compared to the initial state, as the reconstruction precision is largely determined by the enhancement in axial (Z-axis) localization. This addresses a key issue in previous QPM-IFC studies based on hydrodynamic focusing, as described in lines 67-72 of the revised manuscript:

"Axially, cells are either unconstrained or loosely confined, leading to cell dispersion and potential overlap which can compromise single cell identification precision. While digital

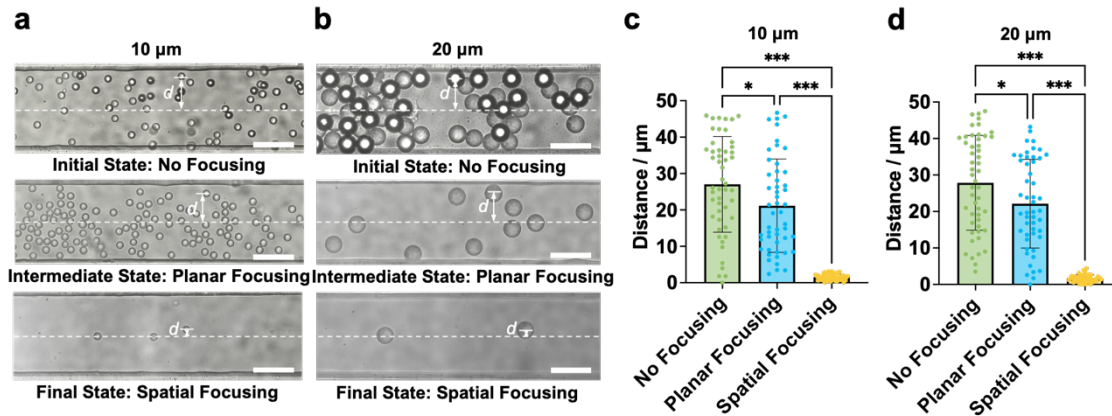
refocusing algorithms can help accurately locate cells at different depths, they are computationally expensive and time-consuming, slowing down IFC's rapid workflow. The lack of axial confinement also causes cell oscillation, imaging artifacts and blurriness, and finally loss of key textures in QPM measurements."

Therefore, our approach significantly improves this aspect, highlighting the enhanced effectiveness and novelty of our method compared to existing studies. Finally, our system achieves a reconstruction time of approximately 0.34 seconds per unit, accounting for only 4.41% of the typical process (~ 7.71 seconds)¹.

ii. Optimizing automated single-cell hologram acquisition

The final state, spatial focusing, is achieved with further constraining the sample flow in the horizontal direction. This step accomplishes the second goal of strictly localizing the single-cell flow to the center of the microchannel. As illustrated in Fig. 2d and e, planar focusing still leaves particles dispersed distributed within the plane. To extract information from individual particles, extensive particle localization and image segmentation are required over a larger field-of-view, which will increase computational complexity and introduce potential errors. In contrast, under spatial focusing, particles are concentrated in a streamlined region at the channel center, enabling direct capture of single-particle images within a fixed central acquisition window ($496 \text{ pixels} \times 496 \text{ pixels}$, $31 \text{ }\mu\text{m} \times 31 \text{ }\mu\text{m}$).

We have now supplemented our analysis with quantitative data on spatial localization by measuring the average distance (d) of the particles' centers from the microchannel centerline. As shown in Supplementary Fig. 11 and Table 6 of the revised Supplementary Information, compared with planar focusing, we find that spatial focusing significantly reduces this distance and tightens the distribution. Specifically, for $10 \text{ }\mu\text{m}$ and $20 \text{ }\mu\text{m}$ microparticles, the average distances of their centers from the channel centerline are $(1.5 \pm 0.8) \text{ }\mu\text{m}$ and $(1.6 \pm 1.0) \text{ }\mu\text{m}$, respectively. The highly efficient spatial focusing not only proves spatial localization precision but also provides a stable and efficient foundation for automated single-cell image acquisition. Additionally, because we can directly capture single-cell holograms at fixed position, localization and segmentation algorithms are unnecessary, further streamlining our phase reconstruction process.



Supplementary Fig. 11 Distance distribution of microparticles under different focusing states. (a) and (b) show the observation of 10 μm (a) and 20 μm (b) polystyrene microspheres in microfluidic channels under difference focusing conditions in white light illumination. d measures the distance of single particle centers from the microchannel centerline. (c) and (d) show the statistical results of the distance under different focusing conditions ($N=50$).

Supplementary Table 6. Distance distribution of microparticles under different focusing states

Focusing State	No Focusing	Planar Focusing	Spatial Focusing
10 μm microparticles	$(27.1 \pm 13.1) \mu\text{m}$	$(21.2 \pm 12.8) \mu\text{m}$	$(1.5 \pm 0.8) \mu\text{m}$
20 μm microparticles	$(27.9 \pm 13.0) \mu\text{m}$	$(22.1 \pm 12.1) \mu\text{m}$	$(1.6 \pm 1.0) \mu\text{m}$

iii. Improving measurement consistency

Furthermore, spatial focusing also improves the consistency of amplitude and phase measurements. As illustrated in Fig. 2h-k, while planar focusing improves accuracy, it still results in higher variability in measurement values. Spatial focusing, through its dual constraints in both vertical and horizontal directions, reduces this variability. We have introduced the Coefficient of Variation (CV), the ratio of the standard deviation to the mean, as an important metric for comprehensively evaluating the precision (accuracy) and stability (consistency) of a measurement system in IFC. Our calculations (see Supplementary Table 7) indicated that the CVs for amplitude and phase measurements reached 3.3%/4.3% (10 μm microspheres) and 1.9%/4.7% (20 μm microspheres) under spatial focusing. These values align with previous studies on the measurement consistency in flow cytometry where CVs are typically $< 5\%^2$, demonstrating the effectiveness of spatial focusing in achieving reliable and

reproducible single-cell analysis. Thus, the quantification of CV values further highlights the necessity of spatial focusing.

In conclusion, we have added more detailed explanations and supporting data to better illustrate these points (see lines 357-405 of the revised Supplementary Information, Supplementary Fig. 11, Table 6 and 7). We have also revised our manuscript to more accurately reflect the necessity of spatial focusing. The specific revisions are as follows:

lines 208-233: Through quantitative comparisons, spatial focusing demonstrates three critical advantages over planar/no focusing. First, it enhances the accuracy and efficiency of amplitude and phase reconstruction. The amplitude-based particle sizes were derived from the mean of measured length/width in reconstructed amplitude maps, while phase-based sizes were calculated via equation (1) using peak-to-valley phase differences (see Methods section). Statistical analysis of 50 microspheres per condition confirmed spatial focusing's necessity for both amplitude (Fig. 2h, j) and phase (Fig. 2i, k) reconstruction. For instance, the phase size values of 10 μm and 20 μm microspheres without focusing were 7.13 μm (Fig. 2i) and 16.5 μm (Fig. 2k), respectively. However, with spatial focusing, these values were refined to 10.4 μm (Fig. 2i) and 19.8 μm (Fig. 2k), closely aligning with their true sizes. This accuracy improvement, coupled with a reduction in reconstruction time (0.34 s vs. 7.71 s per hologram) through eliminated digital refocusing needs (Supplementary Table 5), establishes spatial focusing as a precision-efficiency approach. Second, the strict spatial localization confined particles to a narrow central stream with sub-2 μm lateral deviation (see Supplementary Fig. 11a-d and Table 6 for details), enabling direct imaging through a fixed acquisition window. This approach eliminates the need for particle tracking and segmentation algorithms typically required in planar or non-focusing processes, which can introduce computational complexity and potential misidentification errors, thus laying the foundation for automated hologram capture. Finally, spatial focusing enhanced measurement consistency through flow stabilization. By constraining the particles' trajectory both vertically and laterally, the numerical distribution of amplitude and phase of the particles displayed more compact (Fig. 2h-k). The coefficient of variation (CV), a metric indicative of the precision and stability of hydrodynamic focusing and measurement systems in IFC, was employed to evaluate the platform

comprehensively. Our calculations (see Supplementary Table 7) indicated that the CVs for amplitude and phase measurements reached 3.3%/4.3% (10 μm microspheres) and 1.9%/4.7% (20 μm microspheres) under spatial focusing. These values align with previous studies on the measurement consistency in flow cytometry where CVs are typically < 5%⁶⁶.

We sincerely hope that these revisions and clarifications address your concerns and provide a clearer understanding of the importance of spatial focusing in our study. Thank you again for your valuable input.

References

1. Pirone, D. et al. Speeding up reconstruction of 3D tomograms in holographic flow cytometry via deep learning. *Lab. Chip* 22, 793-804 (2022).
2. Wang, C. et al. Sheathless microflow cytometer utilizing two bulk standing acoustic waves. *Cytometry A*. 99, 987-998 (2021).

2. Following my comment #1, the authors appear to overlook a large body of recent state-of-the-art demonstrations on particle focusing used together with imaging flow cytometry and related applications. These focusing methods could also be applicable to a wide range of particle sizes, with very impressive focusing precision in 2D plane and/or 3D:

- Flow-rate and particle-size insensitive inertial focusing in dimension-confined ultra-low aspect ratio spiral microchannel, *Sensors and Actuators B: Chemical*, 132284, 2022

- Dispersion-free inertial focusing (DIF) for high-yield polydisperse micro-particle filtration and analysis, *Lab Chip*, 2024,24, 4182-4197

- Spiral Large-Dimension Microfluidic Channel for Flow-Rate- and Particle-Size-Insensitive Focusing by the Stabilization and Acceleration of Secondary Flow, *Anal. Chem.*, 96, 4, 1750–1758, 2024

Response: We sincerely appreciate your insightful comments and the references you provided. In our initial manuscript (lines 55-59 of the revised manuscript), we categorized particle focusing methods into active approaches (e.g., electric, magnetic, or acoustic fields) and passive approaches (e.g., hydrodynamic and inertial focusing). However, we did not adequately discuss the specific advancements in this field in recent years. Upon careful examination of the three

papers you recommended, we realized that they all focus on inertial microfluidic techniques, achieving precise inertial focusing of particles or cells within microchannels:

- 1) The study published in *Sensors and Actuators B: Chemical* (2022) developed an ultra-low aspect ratio spiral microchannel that enables flow-rate and particle-size insensitive inertial focusing. When different flow rates (1–3 mL/min) were applied, particles of various sizes (7.3, 9.9, and 15.5 μm) were well-focused near the inner wall (with an efficiency of 90%). Moreover, three types of tumor cells (K562, Hela, and MCF-7) were successfully focused at the same position within the microchannel at higher flow rates (1.5-2.5 mL/min).
- 2) The paper in *Lab Chip* (2024) introduced Dispersion-Free Inertial Focusing (DIF), which achieves 3D focusing of polydisperse microparticles into a thin layer ($<3\ \mu\text{m}$) with a focusing efficiency of over 95%. This technique is applicable to microparticles with diameters ranging from 6 to 30 μm and maintains high efficiency across a wide flow rate range (2.4-30 mL/h). When combined with an ultrafast laser scanning imaging system, DIF enables high-resolution imaging and classification of different cell types.
- 3) The research in *Anal. Chem.* (2024) proposed a spiral large-dimension microfluidic channel with ordered microscopic structures (SLDCOM channel). This design realizes flow-rate and particle-size insensitive focusing. It achieves high efficiency (96%) for particles ranging from 7 to 30 μm and various cancer cells.

Thank you for bringing these references to our attention. **We have now cited these three studies in the context of inertial focusing methods (see references 36-38 of the revised manuscript)**, highlighting their strengths and the potential insights they offer for our research direction. Besides, to further clarify our contributions, we would like to emphasize the following points:

1) Our focus on hydrodynamic focusing-based QPM-IFC

Our primary interest lies in the sub-field of hydrodynamic focusing-based QPM-IFC. As shown in Supplementary Table 16, most comparable studies still rely on hydrodynamic focusing, which is simpler in design and operation. This simplicity is why it has been the predominant and most mature technology in commercial IFC systems for years. Although

inertial focusing has made significant progress, as highlighted in the studies you recommended, it generally requires more complex channel designs (e.g., long bends) and is more dependent on cell physical properties (e.g., size, shape, stiffness, density). We remain open to integrating more advanced particle focusing modules into our platform in the future and look forward to further breakthroughs by our peers in integrating various particle focusing techniques with QPM-IFC. However, our current focus is on addressing challenges within hydrodynamic focusing-based QPM-IFC. **Therefore, we have revised the background section to better contextualize our research. The specific revisions are as follows:**

lines 57-66: In contrast, some passive methods, using special channel geometries (such as inertial focusing^{34, 35}) or fluid properties (such as hydrodynamic focusing) for focusing, are more economical and biocompatible. Recent advancements in inertial microfluidics³⁶⁻³⁸ demonstrate impressive particle focusing capabilities, yet these approaches generally require complex channel designs (e.g., long bends) and are more dependent on particle physical properties (e.g., size, shape, stiffness, density), which may limit their integration with QPM-IFC systems. By comparison, hydrodynamic focusing stands out for its design simplicity, operational convenience, and broad applicability across various particle sizes and flow rates^{39, 40}, along with high compatibility with QPM that facilitates diverse dynamic particle detection.

This focusing helps us address key challenges in hydrodynamic focusing systems, such as axial dispersion and computational overhead, while maintaining compatibility with commercial IFC platforms.

2) Spatial focusing in our QPM-IFC system and its exceptional performance

As detailed in our response to your comment #1, spatial focusing in our QPM-IFC system delivers three synergistic benefits:

- i. Enhances the accuracy and efficiency of amplitude and phase reconstruction**
 - a) Increased reconstructed phase sizes from 7.13 μm (no focusing) to 10.4 μm (spatial focusing) for 10 μm particles, and 16.5 μm (no focusing) to 19.8 μm (spatial focusing) for 20 μm particles, effectively compensating for measurement deviations
 - b) Achieved 22.7-fold faster processing (0.34 s vs. 7.71 s) by eliminating digital refocusing

ii. Optimizing automated single-cell hologram acquisition

- a) Confined particles to a sub-2 μm lateral stream (10 μm particles: $1.5 \pm 0.8 \mu\text{m}$; 20 μm particles: $1.6 \pm 1.0 \mu\text{m}$ from centerline)
- b) Enabled fixed-window imaging without tracking/segmentation

iii. Improving measurement consistency

- a) Reduced amplitude/phase CVs to 3.3%/4.3% (10 μm particles) and 1.9%/4.7% (20 μm particles)
- b) Matched previous studies on the measurement consistency in flow cytometry ($\text{CV} < 5\%$)

These advancements overcome previous limitations of hydrodynamic focusing in QPM-IFC, as elaborated in the revised manuscript.

In conclusion, we hope these revisions and explanations provide a clearer context for our work while emphasizing the unique aspects of our contributions. Thank you once again for your valuable comments.

3. I appreciate the effort on adding a new section of experimental demonstrations - cancer cells detection in human blood. In general, I think this proof-of-principle demonstration provide further evident of how the technique can be used in a scenario closer to clinical setting. In the spike test, the lowest spike ratio is only 1:1000, which is far from the actual CTC application (typically 1:1e5-1e7). Therefore, I would recommend the authors should carefully make their claims - especially in the context of how practical their method can be applied to CTC detection. Also, I think the results in Fig. 7g-j basically showed that the predicted spiked ratio is somewhat consistent with the known ratio. I think without the ground truth of the spiked MDA-MB-231 cells, the authors might not be able to do deeper analysis to quantify the sensitivity and specificity (e.g., ROC analysis). Hence, I would recommend the authors to carefully draw their conclusions. (It seems they didn't experimentally validate the predicted ratio, e.g, Fig. 7g: 507:494).

Response: We are grateful for your positive feedback and valuable suggestions regarding the experimental demonstrations in this section. We appreciate your advice to be cautious in our claims regarding the practical application of our method in CTC detection.

First, we regret the oversight in not explaining the rationale behind choosing the lowest spike ratio of 1:1000 in our original manuscript, and apologize for any confusion this may have caused. As you correctly pointed out, CTCs are extremely rare in whole blood, with counts typically ranging from 1 to 100 CTCs per 10^7 white blood cells (i.e., the ratio is $1:10^5$ - 10^7). However, enrichment techniques, such as microfluidic separation or immunomagnetic bead-based methods, are commonly used to filter out background cells and concentrate CTCs. The spiked ratio corresponds to the purity of CTCs after enrichment, defined as the proportion of CTCs relative to background cells. In light of existing studies, the 1:1000 ratio is reasonable and acceptable when considering the post-enrichment purity ratios reported in the literature¹⁻¹². As shown in Table R1, post-enrichment purity for CTC detection technologies can range from 3.64% (approximately 1 CTC per 27 background cells) to over 90% for advanced enrichment methods. This indicates that our chosen ratio of 1:1000 falls well within the range of experimentally validated post-enrichment scenarios and is much lower than the minimum reported purity of 3.64%. Moreover, the choice of spiked ratios for CTC simulation samples is also based on references to comparable studies^{13, 14}, where post-enrichment ratios are typically permitted to be lower. For example, Suzuki et al.¹³ used a mixing ratio of $(1 \pm 0.15)/16$ (HT29/PBMCs) to simulate CTC-containing blood samples. Therefore, our current ratio has already been lower than the previously mentioned ratio.

Table R1. Best results of purity of the current CTC detection technologies¹

CTC detection technologies	Without enrichment	Negative enrichment	<i>In vivo</i> enrichment	Without ligand capture	With ligand capture-single modality enrichment	With ligand capture-dual modality enrichment
Purity (%)*	-	3.64	-	< 10	84	>92
References	2, 3	4, 5	6	7, 8	9, 10, 11	12

* Purity is the number percentage of the enriched CTCs to the total cells in the enriched samples.

To this end, we have revised the relevant content in the revised manuscript to clarify that our method is designed to work in conjunction with enrichment step, and explain the rationale for our 1:1000 ratio setting. The specific revisions are as follows:

lines 397-399: We created four gradient concentration samples by mixing MDA-MB-231 cells with PBMCs at ratios of 1:1, 1:10, 1:100, and 1:1000. The lowest spike ratio of 1:1,000

was lower than the reported CTC purity ratios after enrichment, which range from 3.64% to over 90%⁷¹.

Second, we fully acknowledge the limitations of our current experimental setup regarding the validation of sensitivity and specificity. While the predicted spike ratios in Fig. 7g-j show consistency with the known ratios, providing preliminary evidence of our method's reliability, we recognize that deeper analyses, such as ROC analysis, are not feasible in our current setting due to the inability to obtain identical samples for ground truth validation. We would like to emphasize that our study is still at the proof-of-concept stage, and we plan to conduct more thorough validation in future research. To address this, **we have revised our manuscript to clearly state the limitations of our current design and eliminate any overstatements regarding clinical applicability. The specific revisions are as follows:**

*lines 27-28: Notably, this system also exhibits high accuracy in **analyzing** simulated ~~clinical~~ blood samples, highlighting its great potential ~~for clinical applications~~ **in practical applications**.*

*lines 399-402: Figures 7g-j illustrate the high consistency between the classification ratios predicted by the LDA model and the actual mixing ratios of the samples, **providing preliminary evidence of our SMHI platform's potential to identify and count** CTCs in **simulated** blood samples.*

*lines 430-435: Finally, we classified and modeled the blood cells to simulate the detection of rare cells in ~~clinical~~ **human** blood samples. By mixing PBMCs and tumor cells in different proportions, our system was able to ~~accurately~~ **effectively** distinguish and count tumor cells in the samples, demonstrating its excellent generalization ability and significant ~~clinical-application~~ **potential for practical application. This proof-of-concept study marks the first step. Further assessment (such as ROC analysis) will be important in future work to fully uncover its advanced performance.***

In conclusion, we have carefully considered your feedback and have made substantial revisions to our manuscript to address the limitations and potential overstatements. We hope these revisions meet your expectations and provide a clearer understanding of our study's contributions and limitations.

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4. Hyun, K. A., Lee, T. Y., & Jung, H. I.. Negative enrichment of circulating tumor cells using a geometrically activated surface interaction chip. *Anal. Chem.* **85**, 4439-4445 (2013).
5. Sajay, B. N. G. et al. Microfluidic platform for negative enrichment of circulating tumor cells. *Biomed. Microdevices* **16**, 537-548 (2014).
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8. Wan, Y. et al. Nanostructured polystyrene well plates allow unbiased high-throughput characterization of circulating tumor cells. *ACS Appl. Mater. Interfaces* **6**, 20828-20836 (2014).
9. Xiong, K. et al. Biomimetic immuno-magnetosomes for high-performance enrichment of circulating tumor cells. *Adv. Mater.* **28**, 7929-7935 (2016).
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13. Suzuki, Y. et al. Label-free chemical imaging flow cytometry by high-speed multicolor stimulated Raman scattering. *Proc. Natl. Acad. Sci.* **116**, 15842-15848 (2019).
14. Zhu, J. et al. Microfluidic impedance cytometry enabled one-step sample preparation for efficient single-cell mass spectrometry. *Small* **20**, 2310700 (2024).

4. Finally, I still have a problem with the “smart” being in the title, because the machine learning methods are the classical supervised learning ones without any new significant improvements. Even though the authors argue that their Optimized feature selection and machine learning model training strategies are different from others, I find it hard to be convincing to say they are novel. Hence, “smart” in this regard appears to be over claimed. I would highly recommend the authors to reconsider the name.

Response: We fully understand and respect your concerns regarding the use of the term "smart" in our title, because this term may imply an overstatement of novelty in our work, particularly in the context of the machine learning methods we have employed.

To address this, we have carefully reconsidered our title. **We have replaced the term "smart" with the more neutral and inclusive term "integrated"**, as we believe it better reflects the core focus of our research on emphasizing the interdisciplinary nature of our work. Additionally, **we have carefully reviewed and revised any overstated descriptions to ensure that our claims are accurately and modestly reflect the contributions of our work.** The specific revisions are as follows:

*lines 21-24: We have developed a high-dimensional phase feature hierarchy and ~~(innovatively proposed)~~ **implemented** a maximum-relevance and minimum-redundancy incremental feature selection (MRMR-IFS) strategy, which effectively addresses feature redundancy and constructs the optimal feature set.*

*lines 103-105: We ~~(innovatively construct)~~ **explore** an incremental feature selection strategy based on the Maximum-Relevance and Minimum-Redundancy (MRMR) algorithm⁶² ranking, which is previously not utilized in the training of advanced machine learning models with phase features.*

We sincerely hope that these revisions address your concerns and provide a more accurate representation of our research. We are committed to maintaining clarity and integrity in our scientific communication and appreciate your guidance in helping us achieve this goal.

Response to Reviewer #3:

Thanks for your expertise and supportive evaluation, guiding us to improve our manuscript.

The response to your comments is list below:

The authors have improved the manuscript significantly and addressed all the reviewers concern adequately. The authors also addressed the novelty, breakthrough in the manuscript. According to me it is an important work for quantitative cell cytometry and addresses an important aspect in the field, specially quantitative analysis of circulating tumor cells. The manuscript is acceptable for the publication in Nature Communication.

Response: Thank you very much for your positive evaluation and support. We appreciate your recognition of our work and look forward to contributing to the field through this publication.

Reviewer #3 (Remarks on code availability):

Not Required

Response: We appreciate your clarification on this matter. We are happy to make the corresponding adjustments and remove the code from the submission if both the editorial team and you consider it appropriate.

Point-by-point Response to Reviewers' Comments

We sincerely appreciate all the Reviewers for their continued evaluation and invaluable comments. Below we submit a point-by-point response letter in which we have provided detailed responses to each comment from the Reviewers and made corresponding revisions in our revised manuscript.

Response to Reviewer #1:

Thanks for your expertise and supportive evaluation, guiding us to improve our manuscript.

The response to your comments is list below:

The authors have chosen to repeat the points already presented in the paper, and answered very lengthy. This does not change the fact that there is no breakthrough in any of the points raised by the authors. Integration of existing methods to something that was existing already is not, to my opinion, suitable to Nat Comm.

Furthermore, the authors claim for several wrong technical novelties. As a specific example, previous works have shown that phase reconstruction can be done much faster than has been shown by the authors. It was already done at 100fps or even more.

Response: We regret that we haven't fully met your expectations. Nevertheless, we're grateful for your feedback. After in-depth analysis and reflection on the reviewers' and editors' comments, we have gained a further understanding of the innovation of our work. We acknowledge that there are still many shortcomings in our research, but the innovative aspects of our work are as follows:

(1) Spatial hydrodynamic focusing plays a crucial role, demonstrating a significant improvement in controlling particle distribution compared to other methods.

(2) Thanks to the precise focusing mode, the speed and accuracy of single-cell phase reconstruction have been greatly improved by eliminating digital refocusing.

(3) It guarantees measurement stability, reducing amplitude/phase coefficients of variation (CVs) to 3.3%/4.3% (10 μ m microspheres) and 1.9%/4.7% (20 μ m microspheres), matching previous studies on the measurement consistency in flow cytometry ($CV < 5\%$).

We believe our work's integrated innovation and precise control system deliver significant improvements to QPM-IFC. Therefore, we maintain that our work aligns with Nature Communications' standards for interdisciplinary research.

Additionally, we'd also like to clarify a potential misunderstanding. The 100 fps refers to the camera's acquisition frame rate, not the phase reconstruction rate. We've already acknowledged the limitation of our current 30 fps in the discussion. Regarding phase reconstruction speed, our current reconstruction time of 0.34 s still shows a significant advantage over comparable studies (7.71s), and we haven't exaggerated this point.

Response to Reviewer #2:

Thanks for your expertise and supportive evaluation, guiding us to improve our manuscript.

The response to your comments is list below:

The authors have addressed adequately all my concerns regarding the strengths and limitations of their method. I have one minor suggestion: Supplementary Fig. S11c-d (Distance distribution of microparticles under different focusing states) should be displayed as part of the main-text figure - it shows significant improvement in controlling the particle distribution compared to other methods, worthing showing in the main text.

Response: We sincerely appreciate your constructive feedback and valuable suggestion. We have now incorporated these panels into Figure 2 of the main text (now designated as Figure 2l-m).

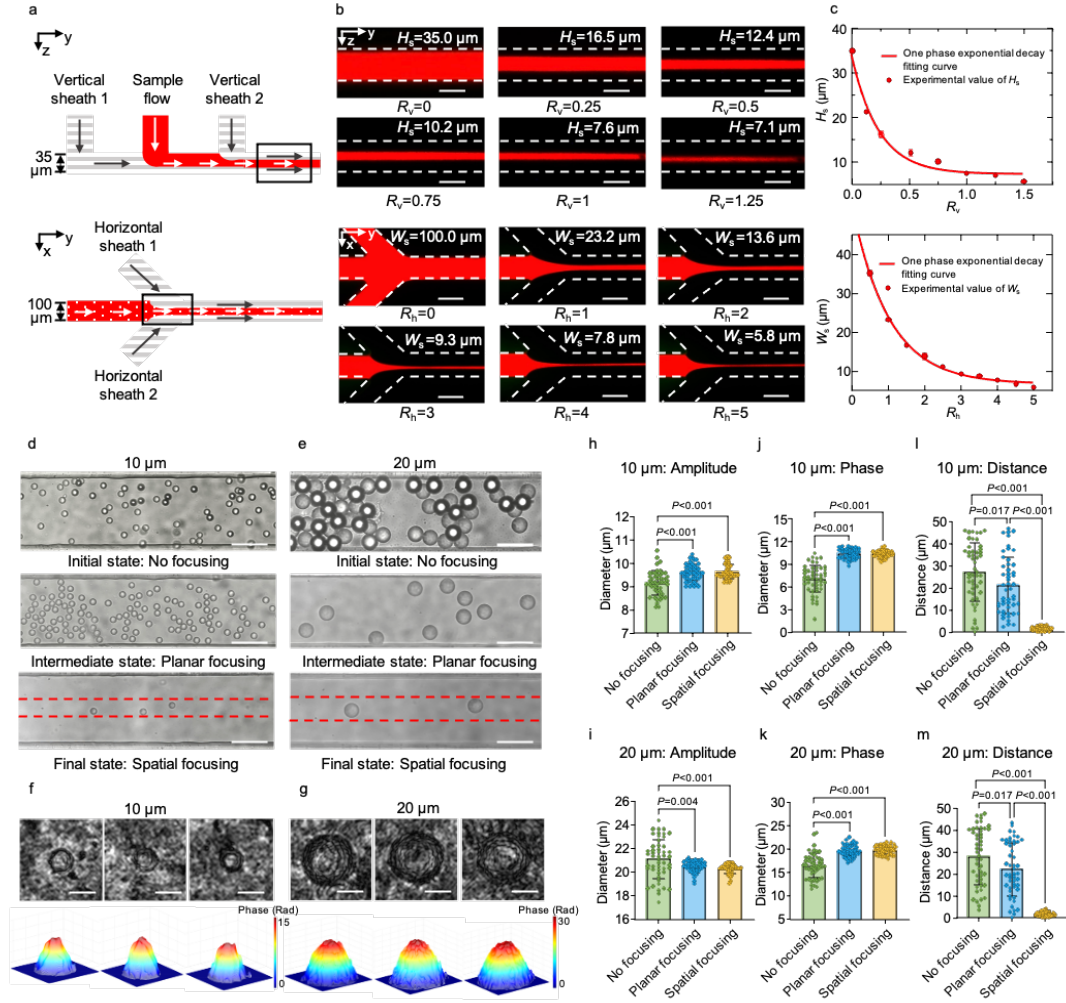


Fig. 2 Spatial hydrodynamic focusing validation and in-focus microparticle DHM detection. (a) Schematic representation of vertical focusing (y-z plane) and horizontal focusing (x-y plane). (b) Experimental results of vertical focusing (y-z plane) and horizontal focusing (x-y plane). The red band represents the sample flow containing fluorescent dye Rhodamine B. The dashed lines represent the boundaries of the microfluidic channels. The sample injection speed is 1 $\mu\text{L}/\text{min}$. R_v represents the velocity ratio of vertical sheath flow to sample flow. R_h represents the velocity ratio of horizontal sheath flow to the combined flows of two vertical sheath flows and sample flow. For horizontal focusing test, R_v is fixed at 0.25. Each focused sample flow height (H_s) and width (W_s) is the mean of 5 independent repeated experiments under the corresponding R_v and R_h conditions. (c) Statistic results of spatial hydrodynamic focusing. It shows the change in H_s with R_v , and W_s with R_h . Data (red dots) are mean values from 5 independent repeated experiments; error bars are SD. (d) and (e) show the flow observation of 10 μm (d) and 20 μm (e) polystyrene microspheres in microfluidic channels under no focusing, planar focusing, and complete spatial focusing conditions in white light illumination. (f) and (g) show the DHM reconstructed amplitude (top) and phase (bottom) maps of 10 μm (f) and 20 μm (g) microspheres under the spatial focusing. The color bar denotes the scale of phase. In (d-g), each microsphere constitutes an independent experimental replicate, and finally amplitude and phase data are collected from 50 independently captured microspheres under each focusing condition for further analysis. (h) and (i) show

the reconstructed particle size of 10 μm (**h**) and 20 μm (**i**) microspheres under different focusing conditions from amplitude. (**j**) and (**k**) show the reconstructed particle size of 10 μm (**j**) and 20 μm (**k**) microspheres under different focusing conditions from phase. (**l**) and (**m**) show the distance from particle center to channel centerline of 10 μm (**l**) and 20 μm (**m**) microspheres under different focusing conditions. In (**h-m**), data are presented as means $\pm$ SD ($n = 50$ microspheres under each focusing condition); the p -value < 0.05 indicates a significant difference decided by one-way ANOVA followed by Tukey's multiple comparisons test. Scale bars: 30 μm (**b**, y - z plane), 100 μm (**b**, x - y plane), 50 μm (**d**), 50 μm (**e**), 10 μm (**f**), 10 μm (**g**). Source data are provided as a Source Data file.