Cell Systems

Transparent Peer Review Record

μDamID: A microfluidic approach for joint imaging and sequencing of protein-DNA interactions in single cells

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Summary

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Scientific editor: Quincey Justman, Ph.D.

First round of review: Number of reviewers: Three
Three confidential, zero signed
Revision invited Oct. 25, 2019
Major changes anticipated
Revision received Jul. 1, 2020

Second round of review: Number of reviewers: Three
Three original, zero new
Three confidential, zero signed
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Data freely available: Yes
Code freely available: Yes

Editor's View

I'm a single-cell person. Ideally, I'm a large-population-of-single-cells-followed-individually-over-time person. I'm motivated by the idea that carefully observing variation and its more directly informative sibling, deviation, may allow us to infer what biology is actually doing and sometimes even why.

I'm also an "orthogonal methods" person. All methods have their own strengths, caveats, and assumptions. Science becomes more powerful when groups of fundamentally different (that is, orthogonal) methods are used to approach the same problem from different angles. The specific perspective each method brings matters.

Altemose et al. rings both these bells. The authors develop microfluidic technology to provide a paired set of observations about single cells: 1) microscopy-based visualization of nuclear lamin-associated domains (LADs) using ^{m6}A-Tracer, a GFP construct engineered to specifically methylated GATC sites, and then also, 2) sequencing-based identification of LADs within single cells using DNA adenine methyltransferase identification (DamID). ^{m6}A-Tracer imaging and DamID have been previously published (an important recent variation: https://www.nature.com/articles/s41587-019-0150-y), but the microfluidic platform Altemose et al. presents to unify them and provide unambiguous, 1-to-1 mapping of one cell's imaging and -seq data is new. That unambiguous, 1-to-1 mapping is door-opening.

Developing technology like this is extremely challenging and both time- and resource-consuming. The hardest part of working with this paper, editorially speaking, was deciding when μDamID had been developed to sufficient maturity to "release it into the wild." It's easy to fall into two traps when thinking about this.

The first trap: It's easy to say, "The proof is in the pudding," which is another way of saying, "If your method is so good, prove it by teaching us something new about biology." I understand this logic, but demonstrating something biologically new, rigorously and deeply, and especially with a new technology, is a Ph.D. project by itself. It's not fair to ask for multiple graduate-student-years in a revision. This sort of thinking also downplays the careful thinking, quality checks, proofs-of-principle that are done along the way, as the technology is designed, tested, and built. Again, unfair.

The second trap: It's easy to arbitrarily add zeros and say "I want hundreds" or "I want thousands" on the assumption that throughput and quality or power go together. An outside expert I consulted re-shaped my thinking on this point. The advice I was given: sometimes a small handful of very high quality observations is more powerful than orders-of-magnitude low quality ones. It depends on the question being asked. To paraphrase my expert, "For the questions my lab asks, we would rather have five exceptionally high quality transcriptomes than hundreds of OK ones." The quality of the information in the five is transformative, even though the quantity is low.

Ultimately, I decided that given the exceptionally high quality of the work presented here, μDamID was ready to be released into the wild when it could generate enough observations to make a statistically sound control-vs.-experiment comparison. There are problems with this standard (for example, it assumes something about effect sizes and prevalence of an observable phenomenon within a population), but to me, it struck the right balance between solidity and optimism: I hope μDamID's paired observations open the door to new insight.

Editorial decision letter with reviewers' comments, first round of review

Dear Aaron,

The reviews are back on your manuscript and I've appended them below. They're split and I'll walk you through my thinking about them.

First, I'll explain the "meta" reason that I was excited about your paper when it came in. We're always looking for pairs of techniques (or more) that have fundamentally different foundational assumptions/guiding principles but report on the same biological phenomenon. We think employing pairs of techniques (or more) is import for cross-validation, sharpening understanding problems, and more generally, for ensuring that important observations aren't missed. Hopefully, it increases the chances that an experiment will surprise you (in a good way). I was excited to see this approach extended to single cells, and particularly to single cells with combination of techniques chosen: imaging and sequencing. Imaging and sequencing data are both feature-rich but in completely different ways, and having the combination in one single, exactly paired experiment is the sort of technology that we hope will open up new biological questions and avenues of experimentation.

This is the main reason that we tend to share the perspectives of Reviewers 1 and 3. Reviewer 2 takes issue with the number of cells analyzed, among other comments. On one hand, this is a fair point, but on the other, it's important to start somewhere. From our perspective, scaling up to seq-like numbers of cells isn't an appropriate goal for this paper, but:

- The benchmarking against scDamID and other demonstrations of quality need to be iron-clad
- The limitations placed on the imaging need to be investigated and described (for example, it's unclear to me whether the cells are alive when they're imaged, and if they're alive, whether they're happy)
- The feasibility of more modest scale-ups needs to be made clear, and
- The scale of these scale-ups (pardon the obnoxious phrasing) needs to be determined by the underlying likelihood of the events you're trying observe in a plausible biological experiment. (This echoes Reviewer 1's comment number 4: how anomalous is anomalous?) If it's unlikely that you'll be able to scale up to e.g. 100's of cells, then the biology you're studying with microDamID needs to be pervasive within the population.

This last one is tricky, because I'd argue that it's hard to discover new biology that occurs frequently and new techniques need to promise, at some level, meaningful discoveries. This is where I see a range of options for this paper going forward. At minimum, if only modest scale-ups are practical, a revision would need to clarify what vLADs are, respond to the reviewers' concerns (details below), and be resubmitted to

us as a 2 figure Brief Report (although note that figures can span full pages and you can have as many supplemental figures as you like). On the other hand, the stronger the scale-ups, the microscopy, and the biology are, the larger and more impactful the final paper can be. (For reference, a full-length article is 7 full page figures.) The strongest revision would make use of the microscopy to pose a question rather than essentially use it as a quality control (i.e. scoring presence/absence of the ring). Here is one idea that you're absolutely welcome to take or leave as you see fit: compare interphase and M-phase cells, which could be FACS-sorted ahead of time.

In addition to these over-arching comments, I've highlighted portions of the reviews that strike me as particularly critical and made some notes inline. Please note that the absence of comments from me doesn't indicate that the comment isn't important! I'd also like to be explicitly clear about an almost philosophical stance that we take at Cell Systems. We believe that understanding how approaches fail is fundamentally interesting: it provides critical insight into understanding how they work. We also believe that all approaches do fail and that it's unreasonable, even misleading, to expect otherwise. Accordingly, when papers are transparent and forthright about the limitations and crucial contingencies of their approaches, we consider that to be a great strength, not a weakness. Please keep this in mind while revising.

As you revise your manuscript, it's important that you and I stay on the same page. I'm always happy to talk, either over email or by phone, if you'd like feedback about whether your efforts are moving the manuscript in a productive direction. Do note that we generally consider papers through only one major round of revision, so the revised manuscript would be either accepted or rejected based on the next round of comments we receive from the reviewers. If you have any questions or concerns, please let me know. More technical information and advice about resubmission can be found below my signature. Please read it carefully, as it can save substantial time and effort later.

I look forward to seeing your revised manuscript.

All the best,
Quincey

Quincey Justman, Ph.D.
Editor-in-Chief, Cell Systems

Reviewers' comments:

Reviewer #1: In "uDamID: a microfluidic approach for imaging and sequencing protein-DNA interactions in single cells", Altemose et al. develop and characterize a microfluidic device that facilitates paired data collection from m6A-tracer and DamID in the same single cells. The work is built upon the DamID technique, pioneered by van Steensel and Henikoff, which leverages deposition of a covalent DNA modification (N6-methyladenosine, m6A) to probe various chromatin phenomena including protein-DNA interactions, transcription dynamics, and chromatin accessibility (van Steensel and Henikoff, 2000;

Southall et al., 2013; Aughey et al., 2018). This technique has previously been adapted for single-cell studies (Kind et al, 2015), however, because of technical limitations the single-cell measurements (scDamID sequencing and m6A-tracer imaging) derive from two separate cell populations. ***[From QJ: this is a very important distinction between your work and previous techniques, so please make sure it's prominently mentioned and highlighted in the figures. It's too easy to gloss over at the moment even though it's intrinsic to everything you're doing.]*** Here, Altemose et al. address this limitation by developing a microfluidic device capable of isolating, imaging, and performing DamID on single cells, thereby allowing scDamID sequencing data to be associated with m6A-tracer data. The authors validate their system against previous work, focusing mostly on the scDamID results of Kind et al, 2015. The validation study involves three main components: (1) proving comparable sequencing read coverage to that of current DamID and scDamID techniques, (2) developing a LAD classifier to be used on single cells and applying it to reproduce previously known associations between LADs and gene density or gene expression, and (3) confirming expected localization of m6A in comparisons of m6A-tracer images and DamID data. In all cases, proper data analyses were applied and presented to demonstrate accordance with previous work. Notably, as is necessary for device/method development, the protocols and information needed to reproduce these experiments are extremely well detailed.

Beyond validation, one observation from this study - that of the 'anomalous' cell - did indeed benefit from the use of uDamID. If the cell were examined by scDamID alone, one would conclude that m6A localized to unexpected genomic regions. With uDamID, the authors were able to clarify that this result occurred because m6A unexpectedly deposited throughout the nucleus. Collectively, the validation studies and results convince me that the device performs as intended in allowing paired single cell imaging and sequencing data. The authors stop short of applying uDamID in other (non-validation) contexts and, e.g. do not use the system to probe new biology.

This paper makes a nice contribution to the exciting, growing body of work in single cell -omics, especially in collecting simultaneous or paired single cell measurements (such as Rooijers et al., 2019). The protocols, device design, and data are well presented, and an informed researcher should be able to use this methodology. In my opinion, the manuscript is appropriate for publication in Cell Systems, after the authors address the following questions/comments.

Comments:

1) Potential limitations of the device appear to be throughput and efficiency. The authors do address throughput in the Discussion, mentioning the use of automated image processing and multiplexed valve control. Nonetheless, the experiments described required three devices to process 25 single cells, which after applying coverage thresholds, resulted in 15 cells analyzed. It is important for the audience to better understand the efficiency of this protocol so that future studies can properly scale up this design in order to address certain research questions. Single cell studies often require a large number of cells (hundreds) to confidently quantify variables like contact frequency or incidence of so-called 'anomalies'. Particularly, comparing with the efficiencies of other single cell DamID studies would help inform a researcher how best to choose an appropriate protocol.

2) While the authors do note that the device is compatible with high-magnification imaging on inverted

microscopes, are there any other limitations on microscopy choices? Previous studies have used super-resolution microscopy methods with m6A-tracer to gain resolution on the scale of tens of nanometers (Kind et al 2015). The lamina is a good and obvious choice; because it contacts a large portion of the genome, low resolution microscopy methods can still reasonably capture the location. However, smaller structures would necessitate higher resolutions. Does the device itself prevent application of other microscopy techniques for any reason (such as having to deal with live cells, bulkiness of pressure system, etc)?

3) In light of previous studies (Kind et al. 2015) applying 100 kb bins for DamID, is there a reason for the authors' choice of 250 kb bins? ***[From QJ: this is also noted by reviewer 2. 100kb bins should be used to facilitate direct, head-to-head comparisons unless that's not appropriate or possible, in which case that should be transparently discussed and could prove to be problematic.]***

4) Interestingly, uDamID has the unique ability to filter cells before scDamID based on imaging quality, as opposed to FACS, which would still collect things like the 'anomalous' cell. How frequently do these 'anomalies' occur? Is it possible that a significant portion of prior scDamID data contains 'anomalies', thus resulting in potential misinterpretations of data? ***[From QJ: Great question, and it'd be great if it could be answered with data.]***

5) The authors use their 11 dam-LMNB1 cells to identify with high confidence a LAD classification termed 'variable LADs' (vLADs). Previous studies have already revealed tissue-specific LADs (facultative LADs / fLADs) from bulk DamID and regions with intermediate nuclear lamina contact frequencies from single cell DamID (Meuleman et al. 2013; Kind et al. 2015). The relationship between these newly classified vLADs and previously identified fLADs or intermediate contact frequency regions is unclear. Can the authors evaluate vLADs within the context of previously identified intermediate LADs, commenting on fundamental differences and potential overlap with those regions? As the single cell DamID from Kind et al. analzyed a different cell line, (the mostly haploid KBM7), it could also be of interest to identify cell-type specific vLADs in addition to the previously characterized fLADs. ***[From QJ: vLADs need to be put in a richer context and characterized more completely. Readers need to be able to understand how they do and don't relate to other LADs.]***

Reviewer #2: Here Altemose et al present a microfluidic approach to simultaneously image and single-cell sequence Lamina-Associated-Domains (LADs). The manuscript describes the implementation of a previously developed microfluidic setup (Street et al., 2014) to image LADs with the m6ATracer method (Kind et al 2013) and of the same cells determine genomic interaction profiles with scDamID (Kind et al 2015). Finally, quantitative and qualitative analyses show that the obtained single-cell data provide specific genome-lamina interaction profiles.

My main concern with this study is that the approach provides little to no technical advantage over current methods. In addition, the method is not implemented to provide any conceptual insights. The number of cells analyzed is very limited (11 after thresholds) and the complexity of the scDamID-profiles is similar to before. With this limited number of cells, combined imaging and sequencing may as well be performed

manually. E.g. combined single-cell m6ATracer imaging and scDamID genomics was performed previously with hundreds of manually sorted preimplantation mouse embryos (Borsos et al., 2019). ***[From QJ: It's worth looking very carefully at this reference. If I'm reading it correctly, Borsos et al. can't draw direct, 1-to-1 relationships between the imaged cells and individual scDamID profiles. If I'm wrong and Borsos et al. does do this, then your revision needs to go farther with respect to demonstrating why microDamID has promise over the methods employed in Borsos et al. (particularly with respect to the microfluidics). If I'm correct, then the ability to draw direct, 1-to-1 relationships between imaged cells and -seq-based profiles is a key advantage of microDamID.]*** Also, the potential conceptual advantage of the combined read-out is not experimentally addressed beyond controlling for cells with the correct DamID-expression levels. In my opinion, this study could potentially be interesting if it allows joint imaging and sequencing of thousands of cells to systematically relate spatial dynamic features to spatial structural organizational states. With the current 10x-genomics sequencing of thousands to millions of cells, sequencing 11 cells appears very limited.

Specific points:

Lines 93-94: "These properties […] on the DNA". Transient interaction will be lost upon DNA replication. DpnI does not digest hemimethylated DNA. Therefore, transient interactions are only permanent in non-dividing cells. This needs to be clearly stated in the text.

Lines 127-129: "[…] for example […] proteins in the nucleus". The text would improve with a more specific example of an example of what can be achieved by combined imaging and genomics.

Lines 136-137: "[…] and we validated […] cell lines". The single-cell data should be more thoroughly and directly benchmarked to the single-cell data from Kind et al., 2015. The choice to focus on cLADs and ciLADs for the analyses is not clear and a more direct quantitative and qualitative analyses are necessary to enable proper evaluation of the quality of the scDamID data. ***[From QJ: this comment contradicts reviewer 1. If you think it's unfair or would like to talk about it further, let me know.]***

Lines 215-217: "We […] Figure 1c". Figure 1c does not provide information on "optimizing expression times".

Lines 231-234: This part comes a bit out of the blue. The necessity of using the V133A is not clear to me. And what is the advantage and rational for using this mutant to perform single-cell DamID?

Lines 248-250: In the methods it is written that the Dam-plamids were "cloned" into Dam-negative bacteria which should result in unmethylated plasmids (line 463). How then are the plasmids a major source of contamination in the sequencing? DamID should not pick it up if the plasmids are not digested by DpnI. Even if this occurs in vivo post transfection, the effects should be limited because most plasmids generally are found in the cytosol (not in the nucleus). ***[From QJ: please clarify.]***

Line 261: The applied bin-size in Kind et al., 2015 is 100kb. Not 250kb. For systematic comparisons (see comment earlier) to Kind et al., 2015 bin-sizes of 100kb should be used.

Line 262: The 15 Dam-LMNB1 cells had median unique fragment count of 110k. Yet, on line 262 only 11 cells are used for the analyses. What is the reasoning to exclude 4 cells? It is not complexity. This needs clarification.

Line 336: As far as I can tell there is no mentioning of "dynamically moving" LADs in Kind et al., 2015.

Figure 3: A track showing single-cell profiles is missing. For a comparison to previously obtained scDamID data single-cell chromosome-wide non-smoothed 100kb binned profiles should be provided.

Figure 4: The ability to filter out transfection-related artifactual cells hardly justifies establishing a microfluidic platform. This work would greatly benefit from more conceptual examples showcasing the advantage of joint

Reviewer #3: The authors have developed a very interesting new technique for joint imaging and DamID profiling of individual cells. While the throughput demonstrated here is modest, it could conceivably be scaled to hundreds of cells. I have two major questions for the authors to consider:

1) Almost all of the library construction steps for DamID are conducted on-chip in the implementation described here (e.g. digestion, ligation, PCR). To what extent is this necessary and beneficial? One can certainly imagine potential benefits including decreased reagent cost or higher reaction efficiency, but there could also be disadvantages such as a more complex chip design that ultimately limits scalability.

2) In Fig. 4, the authors show a relationship between an imaging feature (fluorescence intensity in the lamina vs. interior) and a sequencing feature (signal-to-noise ratio for cLAD vs. ciLAD coverage). It would be interesting to explore the relationship between a broader set of general imaging features and the DamID profiles of the cells (e.g. are there morphological features of the cells or fluorescence intensity distribution that are predictive of the DamID coverage profile for a given cell?).

Authors' response to the reviewers' first round comments

Attached.

Editorial decision letter with reviewers' comments, second round of review

Dear Aaron,

As I mentioned last week, I'm very pleased to let you know that the reviews of your revised manuscript

are back, the peer-review process is complete, and only a few minor, editorially-guided changes are needed to move forward towards publication. Please note that we're happy to publish this work as either an Article or a Methods paper. Both have 7 figures, and the choice is yours (although the paper seems custom built for Methods!). Just let me know.

In addition to the final comments from the reviewers, I've made some suggestions about your manuscript within the "Editorial Notes" section, below. Please consider my editorial suggestions carefully, ask any questions of me that you need, make all warranted changes, and then upload your final files into Editorial Manager. ***We hope to receive your files within 5 business days, but we recognize that the COVID-19 pandemic may challenge and limit what you can do. Please email me directly if this timing is a problem or you're facing extenuating circumstances.***

As you look forward to acceptance, please do consider submitting one of the protocols you've developed in this paper to STAR Protocols, or extending this offer to one of your trainees. STAR Protocols is geared towards trainees and its key purpose is to provide complete and consistent instructions for how to conduct reproducible experiments. If you have any questions, please email starprotocols@cell.com. I'm looking forward to going through these last steps with you. More technical information can be found below my signature, and please let me know if you have any questions.

All the best,
Quincey

Quincey Justman, Ph.D.
Editor-in-Chief, Cell Systems

Editorial Notes

Title: Your title is excellent, but consider being a bit more specific, along the lines of:

μDamID: a microfluidic approach for paired imaging and sequencing protein-DNA interactions within the same single cell

I know that's not quite right because sequencing doesn't happen inside the cell, but perhaps you can come up with a good way of conveying that information? Also, I always suggest that authors check to see if their titles are as effective as they can be. An effective title is easily found on Pubmed and Google. A trick for thinking about titles is this: ask yourself, "How would I structure a Pubmed search to find this paper?" Put that search together and see whether it comes up is good "sister literature" for this work. If it does, feature the search terms in your title. You also may wish to consider that PubMed is sensitive to small differences in search terms. For example, "NF-kappaB" returned ~84k hits as of March, 2018, whereas "NFkappaB" only returned ~8200. Please ensure that your title contains the most effective version of the search terms you feature.

Abstract: I've gone over your abstract to bring it down to our 150 word limit. See what you think. Please feel free to revert anything that you don't like or that you feel distorts your meaning! I apologize if there are instances of the latter.

> *DNA adenine methyltransferase identification (DamID) is a recently developed method which records a protein's DNA-binding history by methylating adenine bases in its vicinity, then selectively amplifies and sequences these methylated regions. These interaction sites can also be visualized using fluorescent proteins that bind to methyladenines. Here, we combine these imaging and sequencing technologies in an integrated microfluidic platform (μDamID) that enables single-cell isolation, imaging, and sorting, followed by DamID. We use μDamID to generate paired single-cell imaging and sequencing data from individual human cells. We map and validate interactions between DNA and nuclear lamina proteins, observe 3D chromatin organization and broad gene regulation patterns, and* ***<insert a phrase about what you learned from the imaging>****. μDamID provides the unique ability to compare paired imaging and sequencing data for each cell and between cells, enabling the joint analysis of the nuclear localization, sequence identity, and variability of protein-DNA interactions.*

Manuscript Text: Your text is excellent, compelling, and clear. Thank you! One very minor thing: "house style" disallows editorializing within the text (e.g. strikingly, surprisingly, importantly, etc.), especially the Results section. These terms are a distraction and they aren't needed—your excellent observations are certainly impactful enough to stand on their own. Please remove these words and others like them. "Notably" is suitably neutral to use once or twice if absolutely necessary.

Figures and Legends: Your figures are excellent, and thanks especially for including such clear and comprehensive figure legends! You have up to 7 full page figures, so please consider moving the more substantive supplemental figures to the main text (e.g. Figs S6 and S7).

Reviewer comments:

Reviewer #1: The authors have done a commendable job of addressing my comments. I do not have any additional comments, and am happy to recommend acceptance of the manuscript.

Reviewer #2: The revised manuscript by Altemose et al has improved considerably. The authors did a solid job in addressing the reviewer's comments. The additional data, analyses/benchmarking and the introduction of the m6ATracer-NES have contributed to a higher quality manuscript. However, my previous concerns about the general conceptual validity of this system to obtain interesting biological insights still stands. As presented, the main advantage of the system lies in the selection of cells with appropriate Dam-LMNB1 expression levels for single-cell analyses. This -to an extend- surpasses the need to generate conditional and stable Dam-expressing cell lines which arguably creates more

experimental flexibility. Yet, in the recent work by Rooijers et al., 2019, hundreds to thousands of cells can be processed in a few days. In my opinion, the investment of creating a stable cell line (and having consistent high quality single-cell profiles), outweighs setting up a non-trivial microfluidics system to select for 50-100 cells with the proper expression levels. Having said that, I do see great advantages for the integration of microDamID and DamID&T. This combination allows linking cellular traits (morphology, LAD dynamics), to cell identity (transcriptomics) and spatial genome organisation (DamID). Perhaps the authors can include few sentences in the discussion about the feasibility and benefits of the integration of these methods.

Reviewer #3: In my opinion, the authors have done a thorough job of responding to my previous questions and have improved the manuscript significantly.

Please find our point-by-point response to the reviewers' comments below, with references to the revisions in the main text, figures, and supplement. Reviewer comments are italicized.

Reviewer #1:

In "uDamID: a microfluidic approach for imaging and sequencing protein-DNA interactions in single cells", Altemose et al. develop and characterize a microfluidic device that facilitates paired data collection from m6A-tracer and DamID in the same single cells. The work is built upon the DamID technique, pioneered by van Steensel and Henikoff, which leverages deposition of a covalent DNA modification (N6-methyladenosine, m6A) to probe various chromatin phenomena including protein-DNA interactions, transcription dynamics, and chromatin accessibility (van Steensel and Henikoff, 2000; Southall et al., 2013; Aughey et al., 2018). This technique has previously been adapted for single-cell studies (Kind et al, 2015), however, because of technical limitations the single-cell measurements (scDamID sequencing and m6A-tracer imaging) derive from two separate cell populations. Here, Altemose et al. address this limitation by developing a microfluidic device capable of isolating, imaging, and performing DamID on single cells, thereby allowing scDamID sequencing data to be associated with m6A-tracer data. The authors validate their system against previous work, focusing mostly on the scDamID results of Kind et al, 2015. The validation study involves three main components: (1) proving comparable sequencing read coverage to that of current DamID and scDamID techniques, (2) developing a LAD classifier to be used on single cells and applying it to reproduce previously known associations between LADs and gene density or gene expression, and (3) confirming expected localization of m6A in comparisons of m6A-tracer images and DamID data. In all cases, proper data analyses were applied and presented to demonstrate accordance with previous work. Notably, as is necessary for device/method development, the protocols and information needed to reproduce these experiments are extremely well detailed.

Beyond validation, one observation from this study - that of the 'anomalous' cell - did indeed benefit from the use of uDamID. If the cell were examined by scDamID alone, one would conclude that m6A localized to unexpected genomic regions. With uDamID, the authors were able to clarify that this result occurred because m6A unexpectedly deposited throughout the nucleus. Collectively, the validation studies and results convince me that the device performs as intended in allowing paired single cell imaging and sequencing data. The authors stop short of applying uDamID in other (non-validation) contexts and, e.g. do not use the system to probe new biology.

This paper makes a nice contribution to the exciting, growing body of work in single cell -omics, especially in collecting simultaneous or paired single cell measurements (such as Rooijers et al., 2019). The protocols, device design, and data are well presented, and an informed researcher should be able to use this methodology. In my opinion, the manuscript is appropriate for publication in Cell Systems, after the authors address the following questions/comments.

Author response: We appreciate reviewer 1's assessment of our study and we have adapted several Figures (Figures 2 & 4, Supplementary Figures 4 & 8) to further highlight the particular

advantage of our system: pairing imaging and sequencing data in the same single cells, and we have emphasized this point in the introduction, results, and discussion (lines 130-133, 423-429, 465-467). Our responses to their specific questions and comments are below.

Comments:

1) Potential limitations of the device appear to be throughput and efficiency. The authors do address throughput in the Discussion, mentioning the use of automated image processing and multiplexed valve control. Nonetheless, the experiments described required three devices to process 25 single cells, which after applying coverage thresholds, resulted in 15 cells analyzed. It is important for the audience to better understand the efficiency of this protocol so that future studies can properly scale up this design in order to address certain research questions. Single cell studies often require a large number of cells (hundreds) to confidently quantify variables like contact frequency or incidence of so-called 'anomalies'. Particularly, comparing with the efficiencies of other single cell DamID studies would help inform a researcher how best to choose an appropriate protocol.

Author response: To help address this question about efficiency more systematically, we ran 4 additional devices with 40 single HEK293T cells that had been transiently transfected with Dam-tdTomato-LMNB1 and m6A-Tracer-NES, and were selected for having a large range of LMNB1 expression based on tdTomato fluorescence. Two cells (labeled D05 and D09) were selected as negative controls, since they had m6A-Tracer-NES signal but lacked laminar rings and had no visible evidence of Dam-tdTomato-LMNB1 expression (Supplementary Figure 4). These cells yielded 41 ng and 145 ng of library DNA, respectively (compared to over 150 ng for most libraries), so we used the lower yield, 41 ng, as a threshold to exclude particularly low-yielding cells from sequencing (except one, labeled D10, to examine what these low-yield library sequences might look like for a cell with a nice laminar ring). In total we sequenced 36 new cells (34 with nice laminar rings and above-threshold yields, D09 with no laminar ring and an above-threshold yield, and D10 with a laminar ring but low yield). Thus, 34 out of 38 cells that were expected from imaging to have high-yield DamID libraries actually produced high-yield libraries (~89%). We have now noted this efficiency in the main text (line 238). By comparison, for conventional in-tube single-cell DamID, 50% of the loaded wells were reported to yield sequenceable libraries (Kind et al. 2015), although there are notable differences in our biological systems, which we describe below (reviewer 2 comment 3). Each of our new sequenced high-yield libraries covered at least 45,000 unique DpnI fragments in the genome (mean 140,000), while the low-yield library covered only 31,000, confirming that filtering on DNA yield is a reasonable screening step prior to sequencing. The overall efficiency of a scDamID protocol is also expected to depend on the amount of DNA in each cell and the expected level of noise due to background methylation, which vary in any particular biological system being studied.

Additionally, our device is designed so that many hundreds of cells can be rapidly screened within the microfluidic channels prior to selection for sequencing, allowing for enrichment of cells with rare phenotypes. The cell inlet port feeds into a large filter area, where dozens of cells are spread out and move at low speeds due to the large cross-sectional area of the filter as they

head towards the narrow channel leading to the cell traps. This allows the user to examine many cells simultaneously in a large field of view using a lower magnification objective. If the pool of cells in the filter area contains a cell with the desired phenotype for sequencing, it can be easily directed to a trap region. If no desirable phenotypes are observed, the entire filter region can be purged and replaced with a fresh pool of dozens of cells in seconds. Thus, even relatively rare visual phenotypes can be selected on the device, provided they are visible at lower magnifications. We have now clarified this advantage in the main text (lines 159-160).

2) While the authors do note that the device is compatible with high-magnification imaging on inverted microscopes, are there any other limitations on microscopy choices? Previous studies have used super-resolution microscopy methods with m6A-tracer to gain resolution on the scale of tens of nanometers (Kind et al 2015). The lamina is a good and obvious choice; because it contacts a large portion of the genome, low resolution microscopy methods can still reasonably capture the location. However, smaller structures would necessitate higher resolutions. Does the device itself prevent application of other microscopy techniques for any reason (such as having to deal with live cells, bulkiness of pressure system, etc)?

Author response: We have now noted in the discussion section (lines 446-458) that µDamID is compatible with most inverted microscope imaging configurations including many common super-resolution microscope configurations enabling, for example STORM, PALM or ground state depletion microscopy. In this study, we harvested live adherent cells in a suspension and imaged their fluorescent proteins with an inverted scanning confocal microscope prior to lysing and processing them for DamID. It would be feasible to run a suspension of previously fixed cells or fixed nuclei through our device, though fixation may affect sequencing efficiency. Imaging methods requiring on-device washing or perfusion steps, like long-term live-cell time-lapse imaging or sequential FISH experiments, are feasible but may require additional fluidics and were not explored in this study. Further, the thickness of the PDMS device presents challenges for imaging methods that require additional condensers with short working distances above the sample.

3) In light of previous studies (Kind et al. 2015) applying 100 kb bins for DamID, is there a reason for the authors' choice of 250 kb bins?

Author response: We initially used 250 kb bins because they are half the median reported LAD length and provided a balance of noise and resolution. We reprocessed all of our original and new data as well as published Kind et al. data in 100 kb bins, which is now reflected in all figures and analyses. This change only decreased our mean classification accuracy by 4%. Our new Supplementary Figure 5a illustrates the distributions of library complexity and LAD classification accuracy achieved with all datasets in 100 kb bins.

4) Interestingly, uDamID has the unique ability to filter cells before scDamID based on imaging quality, as opposed to FACS, which would still collect things like the 'anomalous' cell. How

frequently do these 'anomalies' occur? Is it possible that a significant portion of prior scDamID data contains 'anomalies', thus resulting in potential misinterpretations of data?

Author response: In the revised manuscript we modified our experimental system and extended our sample size to help address these interesting questions. The anomalous Dam-LMNB1 cell in our original dataset had high levels of m6A-Tracer fluorescence, including in the nuclear interior, which we hypothesized was due to high expression of Dam-LMNB1 and m6A-Tracer in that cell, owing to the expression heterogeneity resulting from transient transfection. However, with our existing system we could not test this hypothesis for two reasons: 1) we had no direct readout of the Dam-LMNB1 expression level, which could not be reliably inferred from the m6A-Tracer signal because they were expressed from different plasmids, and 2) we observed that m6A-Tracer localizes to the nucleus even in the absence of Dam methylation, so we could not conclusively determine if high fluorescence in the nuclear interior was due to high methylation or simply due to high m6A-Tracer expression.

To address these two points and to demonstrate the relationship between Dam-LMNB1 expression level and background methylation in the nucleus, we sequenced 36 new cells after making two important changes to our experimental system: 1) we fused the red fluorescent protein tdTomato between Dam and LMNB1, and 2) we attached a Nuclear Export Signal (NES) to m6A-Tracer. Our new m6A-Tracer-NES construct overcomes one of the major limitations of the m6A-Tracer system, which is that m6A-Tracer localizes to the nucleus even in the total absence of Dam. We speculate this is caused by it being small enough to diffuse freely through nuclear pores and having a weak affinity for unmethylated DNA, producing high background fluorescence in cells with low Dam expression levels, low protein-DNA binding site copy numbers, and/or high m6A-Tracer expression levels. This requires a careful ‘tuning’ of m6A-tracer expression for each new Dam fusion protein, limiting the number of cells in a heterogeneously expressing population that have a correct balance of expression for useful imaging. By attaching an NES, we were able to re-localize m6A-Tracer to the cytoplasm in the absence of any Dam, while surprisingly preserving its ability to bind Dam-methylated DNA. We speculate that m6A-Tracer-NES is able to diffuse into the nucleus and is continually exported, unless it binds a methylated target site--a strong interaction that resists removal by exportins. This greatly reduces background fluorescence in the nucleus and expands the versatility of m6A-Tracer for imaging across different expression levels and different Dam fusion proteins. We describe m6A-Tracer-NES in a new results subsection in the main text (starting on line 346) as well as a new main text figure panel (Figure 4a) and a new Supplementary Figure 7.

With m6A-Tracer-NES, excess copies of m6A-Tracer remain in the cytoplasm, providing a more reliable visual readout of where methylated sites actually reside within the nucleus. Simultaneously, the tdTomato fused to Dam-LMNB1 allows us to monitor the localization and expression levels of the Dam fusion protein directly. Even in cells with extremely high Dam-tdTomato-LMNB1 expression, the tdTomato signal was almost entirely excluded from the nuclear interior, producing a bright laminar ring, with excess copies appearing to reside in the cytoplasm. In our expanded sample size, we found no cells with uniform m6A-Tracer-NES signal throughout the nucleus as with the anomalous cell in the original dataset. However, we

trapped and sequenced cells across a range of Dam-tdTomato-LMNB1 expression levels, and we showed that cells with high expression levels tend to have higher background methylation, and thus lower sequencing signal-to-noise or classification accuracy on our positive and negative control sets. These high-expression cells tend to appear more like the original anomalous cell in that they have a more uniform distribution of sequencing reads across the genome (new Figure 4e).

Thus, we speculate that the original anomalous cell likely had both excessive m6A-Tracer expression and excessive Dam-LMNB1 expression, rather than a biological difference in its nuclear organization. Prior single-cell DamID experiments performed in stable, clonal cell lines in which expression levels are expected to be uniform, are less likely to have anomalies like this one with any appreciable frequency. In cases where clonal lines are not possible, using a fluorescent readout of expression level can allow for the user to exclude cells with high expression levels likely to result in high background methylation, and in our system this exclusion can be trivially performed in addition to imaging other molecules or phenotypes at high resolution. Here we used imaging to simultaneously monitor and quantify 1) Dam-LMNB1 expression level using fused tdTomato, 2) LAD methylation using m6A-Tracer-NES, and 3) the shapes and sizes of cells and nuclei using fluorescent and transmission images (Figure 4 and Supplementary Figure 8). We also demonstrated the potential to exclude cells with undesirable phenotypes visible only at high magnifications: cells D05 and D09 had no visible m6A-Tracer rings, which would not be distinguishable by a coarse-resolution method like FACS, but is predictive of poor DNA library quality. We discuss these changes on lines 393-402 and 423-429.

5) The authors use their 11 dam-LMNB1 cells to identify with high confidence a LAD classification termed 'variable LADs' (vLADs). Previous studies have already revealed tissue-specific LADs (facultative LADs / fLADs) from bulk DamID and regions with intermediate nuclear lamina contact frequencies from single cell DamID (Meuleman et al. 2013; Kind et al. 2015). The relationship between these newly classified vLADs and previously identified fLADs or intermediate contact frequency regions is unclear. Can the authors evaluate vLADs within the context of previously identified intermediate LADs, commenting on fundamental differences and potential overlap with those regions? As the single cell DamID from Kind et al. analzyed a different cell line, (the mostly haploid KBM7), it could also be of interest to identify cell-type specific vLADs in addition to the previously characterized fLADs.

Author response: We apologize for this confusion. In our manuscript, to avoid conflation with ‘facultative LADs’, which have been commonly defined as differing *between* cell types, we use ‘variable LADs’ to refer to a conservative subset of LADs that have intermediate contact frequencies *within* one cell type (33-66% contact frequency). To ensure that they do not appear to have intermediate contact frequencies simply due to noise in the data, we use the estimated classification accuracy for each cell to compute the probability of miscalling each region as a variable LAD, and we select only the ~2400 bins with the highest confidence of lying in the

interval (33-66%). We have added text to the results and methods sections (lines 308-314, and beginning on line 849) to clarify this classification.

Specifically, we developed a more broadly useful method for inferring and propagating measurement uncertainty for contact frequency estimates, which will be useful for defining contact frequency confidence intervals in future scDamID studies (now described in Methods lines 851-868 and shown in Figure 3a and Supplementary Figure 6a-c). This method uses our estimates of classification error rates in each bin for each cell to parameterize a Poisson-Binomial model. Using this model one can efficiently compute a probability distribution of the true sample contact frequency in each bin, allowing one to generate confidence intervals and test hypotheses. Furthermore, we can propagate this uncertainty about the sample contact frequency when making inferences about the population contact frequency that account for sampling error. In datasets with large sample sizes and large error, which describes many single-cell datasets, this method avoids producing artificially narrow confidence intervals, which in this study improves our ability to classify regions as variable LADs more accurately.

The reviewer raises an interesting question about the overlap between variable LADs in HEK293T and KBM7 cells. To address this point in the revised manuscript, we investigated the possibility of cell-type specific variable LADs, by comparing contact frequencies between the two cell types. When comparing contact frequencies across the genome we observed high overall correlation (r=0.8), but when looking only at our variable LAD set, we observed a wide distribution of KBM7 contact frequencies (Figure 3d). We took subsets of these regions that vary between the cell types and compared bulk RNA-seq expression levels in those bins, revealing a consistent relationship between gene expression and laminar association on the single-cell level, e.g. bins that have intermediate contact frequency in HEK293T but high contact frequency in KBM7 have lower bulk expression in KBM7 (Figure 3e-g, and Supplementary Figure 6d). These observations validate the differences in contact frequencies seen between cell types and further support the possibility of cell-type specific variable LADs that are related to variability in gene expression. A more rigorous study of cell-type specific variable LADs and their relationship to fLADs would likely require a larger sample of single-cell DamID measurements across multiple cell types. These changes are discussed on lines 333-345.

Reviewer #2:

Here Altemose et al present a microfluidic approach to simultaneously image and single-cell sequence Lamina-Associated-Domains (LADs). The manuscript describes the implementation of a previously developed microfluidic setup (Street et al., 2014) to image LADs with the m6ATracer method (Kind et al 2013) and of the same cells determine genomic interaction profiles with scDamID (Kind et al 2015). Finally, quantitative and qualitative analyses show that the obtained single-cell data provide specific genome-lamina interaction profiles.

My main concern with this study is that the approach provides little to no technical advantage over current methods. In addition, the method is not implemented to provide any conceptual insights. The number of cells analyzed is very limited (11 after thresholds) and the complexity of

the scDamID-profiles is similar to before. With this limited number of cells, combined imaging and sequencing may as well be performed manually. E.g. combined single-cell m6ATracer imaging and scDamID genomics was performed previously with hundreds of manually sorted preimplantation mouse embryos (Borsos et al., 2019). Also, the potential conceptual advantage of the combined read-out is not experimentally addressed beyond controlling for cells with the correct DamID-expression levels. In my opinion, this study could potentially be interesting if it allows joint imaging and sequencing of thousands of cells to systematically relate spatial dynamic features to spatial structural organizational states. With the current 10x-genomics sequencing of thousands to millions of cells, sequencing 11 cells appears very limited.

Author response: We appreciate the referee's detailed feedback on our work. We have revised our study to address the detailed points below, and we believe this has improved our manuscript considerably. However, we disagree with the conclusion that our platform provides no technical advantage over current methods. Using traditional techniques, it is technically challenging to manually isolate a single cell, image that cell with a high-NA objective, and then recover that same cell for genomic analysis. Borsos et al. manually isolate hundreds of cells from preimplantation mouse embryos for DamID, and they do present high-resolution images of some embryos in parallel. However, from what we can gather from the manuscript, they did not produce paired sequencing and imaging data from the same single cells or single embryos. It's also unclear from their methods whether m6A-Tracer was expressed at all in the embryos used for DamID: "For DamID, an mRNA mixture containing 250 ng/ µl Tir1, 50 ng/µl membrane-eGFP and embryonic stage-dependent concentrations of AID–Dam–lamin B1 or AID–Dam were injected into the cytoplasm of oocytes and embryos." In their study there are no data that link LAD profiles with single-cell images. Given their expertise, it could certainly be feasible for them to isolate a single cell from an embryo for paired imaging and sequencing of LADs, but our technology makes this process readily achievable. In the revised manuscript, we scale the number of sequenced cells from 18 to 54, about half of the original single-cell DamID paper from Kind et al. (2015). Here, we emphasize the production of rich imaging and sequencing information across a smaller number of single cells, rather than sparse information across many cells. It should be noted that while 10X Genomics methods can process thousands of cells in a single experiment, image data is inaccessible with this technology. We make the case that imaging provides a means to enrich single-cell populations for phenotypes of interest, potentially reducing the need to sequence thousands of cells before filtering (discussed in detail in the response to reviewer 1 comment 1).

Specific points:

Lines 93-94: "These properties […] on the DNA". Transient interaction will be lost upon DNA replication. DpnI does not digest hemimethylated DNA. Therefore, transient interactions are only permanent in non-dividing cells. This needs to be clearly stated in the text.

Author response: We agree that DamID is most useful for timescales shorter than a cell cycle, though we stop short of saying interactions cannot be detected on longer timescales using

DamID, given that DpnI can cut hemimethylated DNA, particularly in low-salt buffer conditions, though at a lower rate than fully methylated DNA [Sanchez, Marek, and Wangh, *Journal of Cell Science* 1992, PMID 1336780]. We have added a clause to the text to clarify this (now lines 95-96):

“These properties allow even transient protein-DNA interactions to be recorded as stable, biologically orthogonal chemical signals on the DNA, useful for integrating protein-DNA interactions over time, up to the length of a cell cycle.”

Lines 127-129: "[…] for example […] proteins in the nucleus". The text would improve with a more specific example of an example of what can be achieved by combined imaging and genomics.

Author response: We thank the reviewer for this comment and have added another specific application example (now lines 130-133 in the introduction):

“Imaging prior to sequencing also allows for the identification and sorting of complex cytological phenotypes in cells, such as the presence of micronuclei and other nuclear abnormalities that would be difficult or impossible using common fluorescence activated sorting methods.”

and lines 465-467 in the discussion:

“This device could readily be applied to study chromatin organization in micronuclei and other abnormal nuclei by imaging and selectively sorting these nuclear phenotypes and performing DamID, which would be infeasible by bulk or FACS-based methods.”

Lines 136-137: "[…] and we validated […] cell lines". The single-cell data should be more thoroughly and directly benchmarked to the single-cell data from Kind et al., 2015. The choice to focus on cLADs and ciLADs for the analyses is not clear and a more direct quantitative and qualitative analyses are necessary to enable proper evaluation of the quality of the scDamID data.

Author response: Many bulk DamID studies have identified regions of the genome constitutively associated or not associated with the lamina across cell lines (cLADs and ciLADs, Lenain et al. 2017). Furthermore, single-cell DamID data have shown that cLADs tend to have high contact frequencies across single cells, ciLADs tend to have low contact frequencies, and contact frequency is highly correlated with bulk DamID signal (Kind et al. 2015; shown in new Supplementary Figure 5b). We used this prior information to inform our assumption that cLADs with the highest bulk DamID signal are in contact with the lamina in nearly all of our cells, and ciLADs with the lowest bulk DamID signal are not in contact with the lamina in nearly all of our cells. Under this assumption, we defined stringent positive and negative control regions and evaluate the quality of our single-cell data by examining how well we can distinguish these control regions based on their relative sequencing coverage in each cell. This provides a way to

estimate our false-positive and false-negative rates for each cell, which is critical for then defining a stringent set of variable LAD regions that have intermediate contact frequencies. The error rate estimates from these control sets can also be used to parameterize a Poisson-Binomial model allowing us to propagate measurement uncertainty into estimates of sampling uncertainty, providing more reliable confidence intervals around the contact frequency estimate for each bin in the genome (Supplementary Figure 6a-c). We have clarified this further in the Methods section (beginning on line 849).

For a further head-to-head comparison with the Kind et al. 2015 data, we reprocessed 118 of their single-cell DamID sequencing datasets with our same pipeline and plotted their distributions of unique fragments covered as well as their classification accuracy on our positive and negative control regions (Supplementary Figure 5a). We also plot unsmoothed binary LAD calls across chr1 (Figure 2a). Overall, our data span a similar range of unique fragments sequenced, although a subset of our cells show lower classification accuracy for a given number of unique fragments sequenced. We note several important caveats in making such a comparison: 1) Kind et al. sequenced haploid KBM7 cells, while we sequenced hypertriploid HEK293T cells; 2) they expressed Dam-LMNB1 in a clonal stable line, while we used transient transfection, which is expected to produce highly heterogeneous expression levels between cells, and from which we selected many cells with high expression with the expectation that they would have higher background methylation, which we confirmed (Figure 4d); 3) their cells were cell-cycle synchronized while ours were asynchronous, which is a further source of heterogeneity; and 4) our positive and negative control sets were generated in part using filters based on bulk HEK293T DamID data. The greater noisiness of our data is largely expected and attributable to some of these differences in our biological system, rather than an efficiency loss due to using a microfluidic device.

Lines 215-217: "We […] Figure 1c". Figure 1c does not provide information on "optimizing expression times".

Author response: We have modified the text to clarify that the figure panel referred to here provides an example of a laminar ring image (line 203).

Lines 231-234: This part comes a bit out of the blue. The necessity of using the V133A is not clear to me. And what is the advantage and rational for using this mutant to perform single-cell DamID?

Author response: In the revised manuscript, we provide more information to motivate the use of this mutant for single-cell DamID (lines 219-225). We received the Dam-LMNB1 plasmid from another lab and sequenced the plasmid after cloning, revealing that it had acquired this V133A mutation. Given prior literature on this mutant (Elsawy and Chahar 2014), we hypothesized that it might reduce background methylation similar to other Dam mutants (Park et al. 2018), so we decided to try it out for DamID. To test it, we compared it to the wild-type allele in a head-to-head bulk DamID assay and found that the V133A mutant's signal-to-background ratio

(coverage in cLADs vs ciLADs) was over two-fold higher (Supplementary Figure 2), so we decided to use the V133A mutant for our single-cell experiments as well. We note that our expression system involves transient transfection, resulting in heterogeneous gene expression across different cells. It's possible that wild-type Dam can achieve similar methylation specificity in clonal, stable cell lines in which its expression is low and uniform.

Lines 248-250: In the methods it is written that the Dam-plamids were "cloned" into Dam-negative bacteria which should result in unmethylated plasmids (line 463). How then are the plasmids a major source of contamination in the sequencing? DamID should not pick it up if the plasmids are not digested by DpnI. Even if this occurs in vivo post transfection, the effects should be limited because most plasmids generally are found in the cytosol (not in the nucleus).

Author response: This point was clarified in Vogel et al. 2007: "it appears to be impossible to produce unmethylated plasmids in dam− bacteria owing to significant expression of the Dam proteins from the plasmids in the bacterial host". We have shown in unrelated experiments in our lab that while this methylation does still occur in dam- bacteria, it is at lower levels than in dam+ bacteria. So we used dam- bacteria to reduce, but not fully eliminate, plasmid methylation which might contribute to background. The reviewer also raises the interesting possibility that Dam could methylate plasmids in vivo in human cells. It's not immediately clear to us that this would be impossible even in the cytosol. By either mechanism, the plasmid content in each cell is still dwarfed by genomic DNA, and in the vast majority of cells only a small fraction of reads originate from the plasmids (median 0.4%, with only 5/54 cells over 5%; Supplementary Figures 4 and 5a). We suspect high plasmid content to be a sign of low library complexity, rather than a reliable measure of plasmid copy number.

Line 261: The applied bin-size in Kind et al., 2015 is 100kb. Not 250kb. For systematic comparisons (see comment earlier) to Kind et al., 2015 bin-sizes of 100kb should be used.

Author response: We have repeated all analyses with 100 kb bins (see above, Reviewer 1 comment 3).

Line 262: The 15 Dam-LMNB1 cells had median unique fragment count of 110k. Yet, on line 262 only 11 cells are used for the analyses. What is the reasoning to exclude 4 cells? It is not complexity. This needs clarification.

Author response: Cell 007 was excluded due to its anomalous phenotype based on imaging. The other three cells were excluded by a systematic filter removing cells with a high fraction of reads from plasmids (threshold 5%, all three excluded cells were >12%). In two of these cells this high plasmid content was accompanied by a lower number of unique fragments (16k & 10k), consistent with lower library complexity. In re-analyzing our data along with new data, we address the reviewer's concern by filtering based on a more direct readout of library complexity, the unique fragment count itself (shown in Figure 2d).

Line 336: As far as I can tell there is no mentioning of "dynamically moving" LADs in Kind et al., 2015.

Author response: We apologize for this error. We intended to cite Kind et al. 2013, in which m6A-Tracer imaging experiments showed that methylated LADs can move short distances away from the lamina over time. We have corrected this (lines 309-312).

Figure 3: A track showing single-cell profiles is missing. For a comparison to previously obtained scDamID data single-cell chromosome-wide non-smoothed 100kb binned profiles should be provided.

Author response: We have now performed several direct comparisons between our data and scDamID data from Kind et al. 2015 in 100 kb bins across the genome (Supplementary Figure 5a). We now provided the requested plot in Figure 2. We note in a previous comment that there are several important caveats in comparing these datasets due to the different cells and expression systems used.

Figure 4: The ability to filter out transfection-related artifactual cells hardly justifies establishing a microfluidic platform. This work would greatly benefit from more conceptual examples showcasing the advantage of joint

Author response: We thank the reviewer for this comment and have now added additional application examples in the introduction (lines 130-133) and discussion (lines 465-467). We also added new data and analysis that relies on linked imaging and sequencing data. Please see below Reviewer #3 comment 2 for further detail.

Reviewer #3:

The authors have developed a very interesting new technique for joint imaging and DamID profiling of individual cells. While the throughput demonstrated here is modest, it could conceivably be scaled to hundreds of cells. I have two major questions for the authors to consider:

1) Almost all of the library construction steps for DamID are conducted on-chip in the implementation described here (e.g. digestion, ligation, PCR). To what extent is this necessary and beneficial? One can certainly imagine potential benefits including decreased reagent cost or higher reaction efficiency, but there could also be disadvantages such as a more complex chip design that ultimately limits scalability.

Author response: We thank the reviewer for this comment. One can imagine a simplified chip with a single trapping chamber for imaging and an output port that could be connected by tubing

to a conventional PCR plate for scDamID processing. However, it remains challenging to remove a single cell from a microfluidic device and guarantee its delivery to an external tube for processing while avoiding cross-contamination between samples. Although microfluidic platforms do present advantages such as reduced reagent costs, faster thermal cycling, higher gDNA:adapter concentrations, and low lane-to-lane variability in reagent volumes, we primarily chose to perform the scDamID protocol on-device to avoid the issue of cross-contamination between cells and to provide greater certainty that each imaged cell is successfully captured and confined within a lane. We emphasize this now in the main text (lines 149-152). All steps prior to PCR amplification are highly susceptible to losses that may reduce library complexity, so we aimed to keep all input material contained in the device until after the amplification step was complete. We also note that imaging time remains the ultimate limit to scalability. Even in a device with thousands of lanes, if manual image acquisition takes minutes per cell, the practical limit to throughput would be operator time. This could be overcome by automation, although the feasibility of this highly depends on the imaging modality.

2) In Fig. 4, the authors show a relationship between an imaging feature (fluorescence intensity in the lamina vs. interior) and a sequencing feature (signal-to-noise ratio for cLAD vs. ciLAD coverage). It would be interesting to explore the relationship between a broader set of general imaging features and the DamID profiles of the cells (e.g. are there morphological features of the cells or fluorescence intensity distribution that are predictive of the DamID coverage profile for a given cell?).

Author response: To address the reviewer's comment we introduced new imaging information for each cell by fusing tdTomato to Dam-LMNB1 and acquiring red fluorescent images, in addition to green images for m6A-Tracer-NES and a transmission image showing cell size and morphology. We also extracted more quantitative features from each image, including total tdTomato expression, nuclear size, cell and nuclear roundness, and integrated m6A-Tracer signal outside of the lamina. We find that the strongest predictors of library complexity and LAD classification accuracy are nuclear size and Dam-tdTomato-LMNB1 expression level, respectively. These new analyses are included in Figure 4c-e and Supplementary Figure 8 and discussed in the text (lines 393-429).