

ChIP-DIP maps binding of hundreds of proteins to DNA simultaneously and identifies diverse gene regulatory elements

Corresponding Author: Dr Mitchell Guttman

Version 0:

Decision Letter:

8th Feb 2024

Dear Mitch,

Your Article, entitled "ChIP-DIP: A multiplexed method for mapping hundreds of proteins to DNA uncovers diverse regulatory elements controlling gene expression", has now been seen by 3 referees. You will see from their comments below that while they find your work of interest, some important points are raised. We are interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

Reviewer #1 doesn't seem to have major technical issues and says the technique has substantial potential. However, they feel that you have not provided novel biological or mechanistic insights. This concern could be bypassed if you were to reformat this manuscript as a Technical Report (<https://www.nature.com/ng/content>). We encourage you to apply the method to either characterize primary cells and/or transcription factors as suggested, but this is not essential.

Reviewer #2 is mostly positive about this work. Their main points are about technical clarifications.

Reviewer #3 is equally supportive and has some additional technical concerns, which are not damaging.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact me if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

*1) Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available

http://www.nature.com/ng/authors/article_types/index.html here

Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary: <https://www.nature.com/documents/nr-reporting-summary.pdf>

It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.

A revised checklist is essential for re-review of the paper.

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We hope to receive your revised manuscript within ~3-6 months. If you cannot send it within this time, please let us know.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Genetics is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit <http://www.springernature.com/orcid>.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Tiago

Tiago Faial, PhD
Chief Editor
Nature Genetics
<https://orcid.org/0000-0003-0864-1200>

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

In this manuscript, Perez et al. describe an approach (named ChIP-DIP) that utilizes split-pool barcoding to perform chromatin immunoprecipitation for many proteins at once from the same sample. They demonstrate that ChIP-DIP can be scaled to include tens of antibodies per pooled reaction, and that the method is able to generate protein localization maps for cell inputs from ~50K cells/nuclei. The authors apply ChIP-DIP in mouse ESCs and human K562s to show that combinations of histone methylation and acetylation marks often co-occur with one another and can be used to define the regulatory status of specific classes of genes in mESCs. In principle, this is an exciting methodology that should be of great interest to Nature Genetics' broad readership. Our enthusiasm, however, is limited by the manuscript's application of the technology to two "workhorse" cell lines, with minimal novel biological insight. Moreover, we have technical suggestions that should be addressed before publication anywhere. We are hopeful the authors find the below comments constructive.

Major comments:

1. The ChIP-DIP approach seems promising, and we can envision applications where a ChIP-based approach may have advantages over a native tethered-enzyme approach like CUT&Tag. However (as the authors note), there have been several examples of tagmentation-based sample multiplexing published at the bulk and single-cell levels. Can the authors provide some insight into the relative advantages and disadvantages of ChIP-DIP versus recently-published multiplexed CUT&Tag approaches? The authors allude to an inability to profile transcription factors and other chromatin-associated factors with CUT&Tag, but this seems inaccurate given the data recently presented by Brahma and Henikoff (PMID38049663).
2. The authors describe a clever and likely-powerful new method, but then apply the technique to effectively regenerate a subset of the ENCODE / Roadmap Epigenomics data matrix. The largely confirmatory results are thus somewhat underwhelming, especially given the potential of the technique. The authors should perform an experiment that uniquely leverages the throughput of ChIP-DIP to drive biological insight. Some suggestions: performing comparative ChIP-DIP across sorted cells from primary tissue(s) or a tumour model; performing ChIP-DIP following chemical degradation /perturbation of chromatin-associated factors such as cohesin (e.g. Rad21), ATP-dependent chromatin remodelers (e.g. BAF complex), BET bromodomains, or HAT complexes.
3. The authors perform an IgG control antibody ChIP to illustrate the specificity of their antibody-bead coupling. Instead, the authors should consider using antibodies that target mutually exclusive epigenetic marks (e.g. H3K27ac and H3K27me3) to

demonstrate the specificity of ChIP-DIP. In this case, one would expect to uncover genomic domains that should in large part be distinct from one another from a singular mixed experiment.

4. In Figure 2B and 2F, the authors do not specify which values they are using to calculate their Pearson correlation coefficient per condition. Is it the ChIP-DIP signal at peaks called per respective factor per condition?

5. Furthermore, the correlation coefficients appear to be lower for H3K27me3 and RNAPII once the cellular input is reduced <1M cells. Could this be related to the antibody-chromatin ratio that is being used? Are the amounts of antibody used being scaled appropriately based on the number of cells being used in the IP? The authors should comment on whether the approach is robust to the amount of antibody used in addition to the titrated bead pooling detailed in the methods.

6. Comparison of signal levels across antibodies targeting different domains can be confounded by the fact that different antibodies have distinct levels of sensitivity (i.e. using the same peak caller, antibody A could have 2-fold greater number of peaks genome-wide than antibody B). Instead of this density plot in Figure 5D, the authors could split peak lists into quintiles or deciles per antibody and determine the extent to those quintiles or deciles correlate across RNAPI, II, and III-bound loci.

7. In Figure 5A, the authors find certain sets of H3K4me3-marked regions that co-occur with marks associated with active transcription (Set 2) or with heterochromatin (Set 3). However, this does not demonstrate that this association is directly related to "gene type and activity level". The authors could demonstrate that such transcripts at the RNA-seq level are actually distinct from the remainder of the H3K4me3-marked genes to improve this analysis.

Minor comments:

1. The authors argue that chromatin state dictates which loci in the genome can be bound by TFs, RNAPII, and other factors (in final sentences of introductory paragraph). However, many features of chromatin structure (e.g. accessibility) are instead patterned by the binding of such proteins. The authors could improve this section by discussing the possibility that the binding of various TFs and enzymatic factors are what drives accessibility patterns in the genome.

2. For genomic tracks that display binding profiles for the same TF (e.g. Figure 2D), the authors should scale them such that they all depth normalized in the same manner. With these different scales (as indicated by the numbers in square brackets on the right), it is difficult to know if the CTCF antibodies are quantitatively detecting the same level of binding per peak in each condition.

Reviewer #2:

Remarks to the Author:

Perez/Gorozny et al describe ChIP-DIP, a new approach for multiplexed chromatin immunoprecipitation in which many epitopes can be profiled by ChIP in a single experiment. A straightforward split-pool indexing strategy is used to deconvolute the chromatin maps. This method represents a significant leap forward in the realm of multiplexed chromatin mapping strategies, which largely are based on CUT&Tag, because it allows profiling of proteins not well suited to CUT&Tag strategies, like many transcription factors, where CUT&Tag approaches often perform poorly. In addition, the approach allows simultaneous profiling of an unprecedented number of chromatin epitopes. The authors use this approach to identify differences in enhancer and promoters that function in different biological contexts. The method and data are of high quality and the manuscript is well written. There are a few outstanding questions and areas for possible improvement detailed below. There are a few important points made in figures or supplement that need to be discussed in the main text. Some of the 'sanity check' analyses described early in the manuscript are largely confined to histone modifications. However, given the utility of this approach for transcription factor profiling, a similar set of analyses for TFs is warranted. Finally, a deeper exploration of read depth required at different levels of multiplexing would be helpful for future implementation of the approach.

Specific Comments:

1. The experiments testing index cross-contamination in Supplemental Figure 1 are critical for demonstrating the fidelity of ChIP-DIP, but are not meaningfully discussed in the main text. These merit some discussion in the main text and a clearer description in the Supplemental Figure or Legend. (The experiments are well explained in the Supplemental Note, but this could be missed by some readers.)

2. My expectation is that read depth would need to scale in proportion to the number of antibodies utilized in an experiment, but it is not clear whether this was tested by the authors. This point could presumably be addressed by down-sampling deeply sequenced datasets derived from different levels of multiplexing and assessing the quality of individual maps at each read depth (peak number, correlation with benchmarking datasets, etc).

3. The authors perform a series of comparisons between ChIP-DIP and ENCODE datasets for mostly histone modification data in Fig. 1B-E. Since one major advantage of ChIP-DIP over other approaches is its utility for multiplexed TF mapping, it would be very useful to see similar analyses for some TFs, where high quality published ChIP datasets are available.

4. The authors write "For example, we found that RNAP I and II promoters display stronger H3K56Ac enrichment and RNAP I and III display stronger H3K4me2 enrichment relative to H3K4me3 (Figure 5D)." Although these patterns are interesting, it

can be difficult to form conclusions about the relative levels of histone marks measured by ChIP, which necessarily uses different antibodies. The maps are normalized for read coverage, but may reflect very different absolute abundances of these marks at individual promoters, due to differences in ChIP efficiency, etc. I suggest modification of the text to reflect the inherent limitations of these comparisons.

5. Minor: Fig. 1A – the figure should be modified to reflect sheared crosslinked chromatin is used as an input, not crosslinked cells.

6. Minor: Fig. 1B – it would be useful to also see an aggregate track for the ENCODE reference data used in the figure. It may or may not look similar to the ChIP-DIP “All Reads” track.

7. Minor: In Figure 5B, the authors note differences in the patterns of histone marks near transcription start or end sites of RNA Polymerase I, II, and III genes. Are these differences due to differences in promoter bidirectionality, which is common for Pol II genes? This could be addressed if a suitable nascent transcript sequencing dataset were available.

Reviewer #3:

Remarks to the Author:

Summary: Perez et al. describe ChIP Done In Parallel (ChIP-DIP), a method for conducting ChIP-seq chromatin profiling on a large catalog of targets simultaneously from the same cellular material in the same experiment. The linchpin of the technology is a mixture of paramagnetic beads that pair antibodies with antibody-specific sequencing barcodes, which can be individually identified in a complex mixture with several successive rounds of barcoding via combinatorial indexing. In this way, beads harboring antibodies for several different targets can be combined in the same ChIP-seq experiment. The authors demonstrate that ChIP-DIP yields highly accurate ChIP-seq profiles that are of comparable quality to ENCODE ChIP-seq data. They go on to show that over 100 targets can be co-profiled in the same experiment. By analyzing co-occurring patterns within these data, the authors are further able to characterize distinct chromatin states at initiation sites for RNA polymerase I, II and III, and distinguish apparent gene and enhancer regulatory subtypes with unique combinations of histone acetylation. In all, the authors demonstrate a compelling technological adaptation that promises to make “consortium-scale” ChIP-seq possible in a single lab.

Evaluation: This is a clever method, and the controls for data quality appear to be thoughtful, careful, and well conducted. However, there are more subtle sources of potential background that might be worth addressing, including cryptic multi-bead interactions. I also think there are a couple technological clarifications that should be made for the benefit of potential users of ChIP-DIP: First, how the yield and data quality compare with “real” input cell numbers, and second, how ChIP-DIP compares with alternative techniques such as CUT&RUN and CUT&Tag, for which high-throughput, “low-touch” approaches are already available.

Impact: I agree with the authors that this technology, if sufficiently robust and controlled for, offers the potential for “consortium scale” ChIP-seq in a single or a few experiments. Such an accomplishment would democratize large-scale ChIP-seq in a way that hasn’t really been possible previously. Although it is worth weighing its utility against non-ChIP-seq alternatives that have accomplished some of the same goals, this work should still be of broad interest to the chromatin and gene regulation fields.

Major Comments:

- One potential source of background the authors have not explicitly considered here is the binding of a single particle by two beads. This could be a particularly insidious source of background because it may manifest in multivalent “stickiness” where one or a few “true” co-modified particles that gets bound by two different beads could seed the association of many other particles that wouldn’t otherwise associate. Although the correlations shown throughout this manuscript show that there is good specificity of targets on a genome-wide level, one could imagine this phenomenon subtly degrading the specificity at the locus level in a way that would be difficult to detect but could impact downstream analyses. A few points here:

o The concern here is partly addressed in the human-mouse mixing experiment, but it’s hard to know to what degree “bead stickiness” is contributing since this phenomenon would be indistinguishable from a violation of the “birthday problem” in which two distinct particles with different antibodies get the same barcode combination, and the authors haven’t articulated what the expected mixing rate is here based on the number of input particles vs. possible barcode combinations. Can the authors comment on the degree to which birthday problem effects vs. bead stickiness effects are at play here? If the authors were to reduce the input relative to the possible barcodes, would mixing rate go down (indicating a birthday problem) or would it stay roughly the same (indicating a bead stickiness problem)?

o If observed mixing is 4-6%, actual mixing is expected to be double that due to unobserved intra-species mixing, and so the real mixing rate (8-12%) should be reported, especially since it is actually rather high as compared to many other combinatorial indexing-based single-cell profiling approaches.

o mESCs might be expected to suffer from bead stickiness more acutely since bona-fide H3K27me3-H3K4me3 bivalent domains might seed apparent H3K27me3-H3K4me3 co-occurrence that doesn’t actually exist. Can the authors show explicit comparisons, both genome-wide and on the locus level, between mESC H3K27me3 and H3K4me3 ENCODE ChIP-seq and ChIP-DIP to demonstrate this isn’t the case? Both genome-wide correlation heatmaps and locus-specific browser shots

for bona fide bivalent and non-bivalent regions as defined by ENCODE ChIP-seq would seem appropriate here.

- Lines 70-72: While I appreciate the potential advance represented by this technology, this particular sentence acts as though time has stood still since ENCODE. There has been a decade of technological improvements since then, including CUT&RUN and CUT&Tag that are now in widespread use precisely because of their utility across a range of targets and their proficiency on the productivity metrics the authors are trying to address. According to the 2018 Janssens et al. paper cited here (#19), high-throughput automated CUT&RUN can be implemented for \$75/sample in two days per 96 samples. If we're substantially less generous and call it \$150/sample in 4 days, to recreate the work listed in Supplementary Table 1 (614 samples by my count) would cost \$92,100 and take ~1.5 months in a single lab. How does ChIP-DIP compare to these? Given that the authors cite cost, manpower, and time as important factors upon which ChIP-DIP improves, it would make sense to compare exact numbers for those variables with this and/or other up-to-date alternatives so that potential users can make an informed decision about the comparative advantages of this approach.

- Lines 135-140: I think it's worth considering the number of effective cells that are actually being profiled here. It is well established that combinatorial indexing-based methods for single-cell molecular profiling experience significant cellular loss relative to input. This is owing to both the loss of cells/DNA molecules through buffer exchanges and molecular processing steps, and the fact that only a very small proportion of the possible barcode combinations available end up being used. Therefore, it seems as though the "cells/target" calculated for Fig. 2E is likely optimistic, and the user can't necessarily be confident that the profiles they recover are truly equivalent in quality and library complexity to what would have been obtained from profiling the equivalent number of cells in isolation via ChIP or CUT&RUN/Tag. Could the authors provide some demonstration of the quality (e.g. FRIP) and estimated library size (e.g. via PicardTools) of their ChIP-DIP profiles that they can benchmark to a range of ChIP profiles from different numbers of cells so that a more accurate estimate of effective cells profiled could be ascertained? This could probably be accomplished without extra experiments by simply downsampling a single individual ChIP-seq experiment to represent different effective cells profiled and comparing it to ChIP-DIP.

Minor comments:

- Lines 58-60: Just to note that there are actually quite a few methods for multifactorial chromatin profiling that have been developed in the past few years. In addition to the ones cited here, there are also MulTI-Tag (Meers et al. Nat Biotech. 2022), NTT-seq (Stuart et al. Nat Biotech. 2022), and uCoTarget (Xiong et al. Sci Adv. 2024, and on bioRxiv since April 2023). However, all of these technologies also have the benefit of detecting bona-fide co-occurrence of histone modifications in the same single cells, which ChIP-DIP does not as presented here, so the comparison is somewhat apples and oranges. The more appropriate comparison for ChIP-DIP would probably be with high-content ChIP or CUT&RUN/Tag approaches (as outlined above).

- Figure 4: Accurate transcription factor profiling has always been challenging and the array of TFs profiled here is impressive, but there should be quality metrics reported for every transcription factor included here, for instance the rank of the TF's expected binding motif in the top recovered motifs, and the percentage of fragments containing an expected binding motif.

- How is the ChIP-DIP target-specific yield dependent on the other species added to the pool? I'm particularly concerned that highly similar epitopes, e.g. different methyl valency states of the same modification, might promiscuously bind to each others' antibodies when they are both in the mix, especially since it's known that many histone modification antibodies can have low specificity (for instance, as catalogued here: <https://chromatinantibodies.com/>). Have the authors evaluated how correlations change for the same ChIP-DIP target when it's profiled in concert with different partner targets in the same pool?

- Figure S7: Histone acetyl antibodies in particular are known to be highly promiscuous. Do the co-modified clusters you identify here line up with what has been found from mass spectrometry studies of co-modified histone tails (to the extent that they exist)?

Version 1:

Decision Letter:

Our ref: NG-TR64182R

14th Aug 2024

Dear Mitch,

Thank you for submitting your revised manuscript entitled "ChIP-DIP: A multiplexed method for mapping hundreds of proteins to DNA uncovers diverse regulatory elements controlling gene expression" (NG-TR64182R). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Genetics, pending minor revisions to comply with our editorial and formatting guidelines.

We will be now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements soon. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Genetics. Please do not hesitate to contact me if you have any questions.

Congratulations!

Sincerely,

Tiago

Tiago Faial, PhD
Chief Editor
Nature Genetics
<https://orcid.org/0000-0003-0864-1200>

Reviewer #1 (Remarks to the Author):

The authors have done an admirable job of addressing all of our comments. This manuscript will be of great interest to Nature Genetics' readership.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, the authors include many new analyses and data, thoroughly addressing my comments. The manuscript is substantially improved. I have no additional concerns.

Reviewer #3 (Remarks to the Author):

I thank the authors for their conscientious consideration of my concerns and recommendations, and I find that they have adequately addressed them.

made.

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Reply to Reviewers' Comments

We thank the reviewers and the editor for their careful reading of the manuscript and helpful suggestions, which we believe have greatly improved the manuscript. We are delighted that all of the reviewers find our work to be important; they raised several technical questions that we have addressed in the revised manuscript. In addition, based on the recommendation of the editor and reviewers, we have added additional data profiling chromatin dynamics upon stimulation of primary mouse for primary mouse cells (Bone marrow dendritic cells stimulated with LPS. We highlight these changes in detail in the point-by-point response below.

Reviewer #1

In this manuscript, Perez et al. describe an approach (named ChIP-DIP) that utilizes split-pool barcoding to perform chromatin immunoprecipitation for many proteins at once from the same sample. They demonstrate that ChIP-DIP can be scaled to include tens of antibodies per pooled reaction, and that the method is able to generate protein localization maps for cell inputs from ~50K cells/nuclei. The authors apply ChIP-DIP in mouse ESCs and human K562s to show that combinations of histone methylation and acetylation marks often co-occur with one another and can be used to define the regulatory status of specific classes of genes in mESCs. In principle, this is an exciting methodology that should be of great interest to Nature Genetics' broad readership. Our enthusiasm, however, is limited by the manuscript's application of the technology to two "workhorse" cell lines, with minimal novel biological insight. Moreover, we have technical suggestions that should be addressed before publication anywhere. We are hopeful the authors find the below comments constructive.

We are delighted that the reviewer finds the ChIP-DIP method to be an exciting methodology that would be of great interest to a broad audience. We appreciate their detailed and thoughtful review and address their specific questions below.

Major comments:

1. The ChIP-DIP approach seems promising, and we can envision applications where a ChIP-based approach may have advantages over a native tethered-enzyme approach like CUT&Tag. However (as the authors note), there have been several examples of tagmentation-based sample multiplexing published at the bulk and single-cell levels. Can the authors provide some insight into the relative advantages and disadvantages of ChIP-DIP versus recently-published multiplexed CUT&Tag approaches? The authors allude to an inability to profile transcription factors and other chromatin-associated factors with CUT&Tag, but this seems inaccurate given the data recently presented by Brahma and Henikoff (PMID38049663).

We thank the reviewer for their enthusiasm regarding the promising nature of ChIP-DIP.

We agree that, as with any method, ChIP-DIP has advantages and disadvantages for different applications. Our goal is not to diminish the importance and value of existing

multiplexed CUT&Tag methods but rather to highlight the specific utility of ChIP-DIP as it relates to the existing needs in the field and the reported limitations of existing methods.

Although there have been important improvements in alternative methods, the current literature clearly highlights two limitations with multiplexed Cut&Tag methods: scale and protein target diversity (see references Gopalan, 2021; Kaya-Okur, 2020). ChIP-DIP overcomes both of these:

- 1) **Scale**: Previous multiplexed CUT&Tag methods have generated data for at most 3 (Gopalan, 2021; Gopalan, 2022) proteins. While this distinction might appear to be one simply of the scale tested, based on what is reported in those papers and from our discussions with the authors (who are actively working with us to integrate our ChIP-DIP barcoding scheme with multiplexed scCUT&Tag to expand scale) this limitation is currently an upper limit on the multiplexing capability. Specifically, the Gopalan et al. 2021 multi-CUT&Tag paper states that:

"the efficiency of multi-CUT&Tag is likely limited by the fact that only two Ab barcodes can be present in each unique genomic insert. Accordingly, if a very high number of different AbdpA-Tn5 combinations are used, AbdpA-Tn5 complexes corresponding to co-localizing chromatin proteins will likely interfere with each other by competing for insertion into the same DNA fragments."

In contrast, we showed that ChIP-DIP is able to generate high-quality datasets within pools containing >100 distinct antibodies, including within pools where multiple antibodies target the same protein or multiple proteins within the same complex. Because of the nature of the approach, we believe that that this does not reflect an upper limit and that it would work to multiplex even larger numbers of proteins.

- 2) **Diversity of protein types**: Numerous published reports, as well as product literature from companies that sell CUT&Tag kits, have highlighted that CUT&Tag is less amenable to low abundance targets, such as transcription factors and chromatin regulators, and performs best for histone modifications. For example, the CUT&TAG-IT assay kit produced by ActiveMotif is marketed as "*Rapid and robust genome-wide analysis of histone marks at lower sequencing depths*" and compatible targets described as "*primarily histone modifications*". Similarly, the CUTANA™ CUT&Tag Kit produced by EpiCypher is marketed as "*CUT&Tag provides robust profiling for histone PTMs*." Finally, the CUT&Tag Assay Kit from Cell Signaling Technologies states that "*CUT&Tag works very well for analyzing histone modifications*".

The creators of multi CUT&Tag also note this diversity limitation in their Gopalan et al. 2021 paper, stating that:

"further work is necessary to enable robust multi-CUT&Tag profiling of sequence-specific DNA binding proteins, such as transcription factors, due to their poorer performance relative to histone modifications and RNAPII in CUT&Tag"

Nonetheless, the reviewer is correct that in some specific cases CUT&Tag can work for some TFs or CRs, but these examples are currently the exceptions. The Brahma and Henikoff paper highlights this exact point: while the authors indeed were able to generate high-quality maps for two non-histone targets (RNAPII and BRG1) using CUT&TAG, they were not able to map other cell-type specific TFs such as Nanog, Oct4, KLF4 and Sox2. Indeed, for these TFs, the authors instead used CUT&RUN. This is certainly not a critique of the paper; CUT&RUN is an important method, but we note this because CUT&RUN has not yet been – and likely cannot be – multiplexed in the way that CUT&Tag has been.

In contrast, using ChIP-DIP, we were able to map all of these factors RNAPII, BRG1 as well as Oct4, Sox2, and Nanog along with dozens of other chromatin-associated proteins within a single experiment.

In the revised manuscript, we have reworked the Introduction to clarify these key needs (scale and diversity) (page 3) and added a section to the Discussion comparing ChIP-DIP to these other methods (page 16).

2. The authors describe a clever and likely-powerful new method, but then apply the technique to effectively regenerate a subset of the ENCODE / Roadmap Epigenomics data matrix. The largely confirmatory results are thus somewhat underwhelming, especially given the potential of the technique. The authors should perform an experiment that uniquely leverages the throughput of ChIP-DIP to drive biological insight. Some suggestions: performing comparative ChIP-DIP across sorted cells from primary tissue(s) or a tumour model; performing ChIP-DIP following chemical degradation /perturbation of chromatin-associated factors such as cohesin (e.g. Rad21), ATP-dependent chromatin remodelers (e.g. BAF complex), BET bromodomains, or HAT complexes.

We thank the reviewer for these suggestions.

The primary goal of our manuscript was to present and validate a new method that we believe will be of enormous utility to the larger community for many different applications. To achieve this goal, we set out to demonstrate that ChIP-DIP can generate high-quality data that accurately recapitulates the existing ‘gold standard’ datasets. This was why we focused on generating data that was already extensively profiled and validated by the ENCODE and Roadmap Epigenomics projects and highlighting the high-level of concordance between these datasets.

While we agree that ChIP-DIP has the potential to be used in many distinct biological systems and reveal many novel biological insights, that is not the goal of this paper. Nonetheless, we appreciate the importance of demonstrating that our approach can be used in other cell contexts, such as primary cells. To demonstrate this, we have now generated a dataset corresponding to 24 proteins (22 histone modifications, CTCF and HP1 β) within a time course of primary bone marrow derived dendritic cells stimulated with LPS.

Briefly, we show that:

- Multiple chromatin modifications, including modifications demarcating active promoters, gene bodies, enhancers, insulation domains and repressive domains, change substantially across the time course.
- We explore the relationship between transcription and histone modifications at promoters, gene bodies, or enhancers, noting that transcriptional changes are more strongly linked to enhancer acetylation changes than promoter or gene body histone changes.
- We explore the genome-wide changes in histone acetylation (H3K27ac) and find three general patterns across time, with a set of regions unchanged, a set of regions newly acetylated and a set of regions deacetylated post stimulus, corresponding to stable, induced and repressed genes, respectively.
- Finally, we explore the set of newly acetylated regions and the corresponding transcription over time and note three distinct temporal patterns – a “burst” group that is intermittently acetylated and transcribed by 6hr, but deacetylated by 24hrs, a “delayed” group that is only acetylated and transcribed after 6hrs, and a “steady” group that is fully acetylated by 6hrs but whose transcription continues to increase up to 24hrs.

This additional dataset further highlights the broad utility of our method for use in different experimental contexts, including in primary cells and across time. In addition, this application highlights an additional important technical capability of the ChIP-DIP method: we can generate maps across multiple experimental conditions within the same multiplexed experiment. We present these data in Figures 5 and S15 and describe the results on pgs 10-11.

3. The authors perform an IgG control antibody ChIP to illustrate the specificity of their antibody-bead coupling. Instead, the authors should consider using antibodies that target mutually exclusive epigenetic marks (e.g. H3K27ac and H3K27me3) to demonstrate the specificity of ChIP-DIP. In this case, one would expect to uncover genomic domains that should in large part be distinct from one another from a singular mixed experiment.

We apologize for our lack of clarity in explaining the goals of this specific experiment.

To clarify, the experiment used to demonstrate antibody-bead specificity (shown in Supplemental Figure 1D) aimed to understand how often an antibody that was bound to a labeled bead might dissociate and reassociate with a different bead containing a distinct oligo. The reason this is important is because this behavior may lead to incorrect assignment of a protein to DNA.

To measure dissociation, we used chromatin as a readout of antibody movement between distinct labeled beads within the pool. Specifically, we wanted to explore whether chromatin that was IP'ed with one antibody ends up on a clearly distinct bead because the antibody dissociated from the bead and reassociated with another protein G bead in solution.

To do this, we performed an IP using beads containing an antibody against a protein that binds chromatin (CTCF) and then mixed these beads with oligo-labeled beads that lacked any antibody (“empty” beads). If CTCF antibodies dissociate and rebind other beads during ChIP-DIP, we would detect chromatin on “empty” beads. However, we rarely detected chromatin on empty beads, indicating that CTCF antibodies do not dissociate and rebind to other beads during the ChIP-DIP procedure. In this experiment, IgG was simply used as another bead-type that would be expected to lack chromatin associations (“empty”) but is otherwise of no particular significance. We have clarified the goal and description of this experiment on page 5.

While we appreciate the reviewer’s suggestion to use antibodies that target mutually exclusive marks, this does not get at the question of dissociation directly because incorrect chromatin assignment will also be additionally confounded by the specificity of each antibody rather than representing a direct readout of the antibody-bead relationship. Nonetheless, ChIP-DIP does clearly separate mutually exclusive chromatin domains demarcated by functionally distinct histone modifications, including H3K27ac and H3K27me3. We present this data in Supplemental Figure 10.

4. In Figure 2B and 2F, the authors do not specify which values they are using to calculate their Pearson correlation coefficient per condition. Is it the ChIP-DIP signal at peaks called per respective factor per condition?

We apologize for the lack of clarity in our initial description of the values used in Figure 2B and 2F. To clarify, we generated a set of regions defined as the union of all peaks called for any of the four targets in the experiment with the greatest sequencing depth per target (10Ab K562 Experiment). For each region, we computed the background corrected ChIP-DIP signal for each sample (see Methods pgs. 73-74 for details on background correction). We then computed the Pearson correlation coefficients across all regions between all pairs of samples and plotted this as a heatmap. We have updated the figure legend to clarify this process (pgs. 22-23).

5. Furthermore, the correlation coefficients appear to be lower for H3K27me3 and RNAPII once the cellular input is reduced <1M cells. Could this be related to the antibody-chromatin ratio that is being used? Are the amounts of antibody used being scaled appropriately based on the number of cells being used in the IP?

To minimize the number of variables and test the robustness of ChIP-DIP to varying cell input numbers, we used the same bead pool for all conditions. As such, the total amount of antibody and number of beads that we used were the same for all cell inputs.

The primary reason for the observed reduction in the correlation coefficients at lower cell input levels appears to be due to lower chromatin yield resulting in fewer peaks detected rather than the antibody-chromatin ratio used.

There are two possible reasons that the correlation over the union of peak regions for all four targets could decrease in such an experiment:

- 1) **Specificity:** Reduced cell input results in higher antibody to chromatin ratios, which could lead to an increase in non-target binding. This would be observed as an increase in the number of false-positive peaks detected at lower cell numbers. However, in practice, we do not see this. While we observe fewer significant peaks with lower cell input numbers, almost all are also observed in the higher cell number. For example, for virtually all proteins – including CTCF, H3K4me3 and H3K27me3 – we observed that >90% of peaks detected at 5×10^4 cells are also detected as peaks at higher cellular inputs. We report these overlap statistics in Figure S7.
- 2) **Sensitivity:** Having less cell input might lead to lower yield and detection of on-target protein capture. This would be observed as a decrease in the amount of chromatin captured for each given protein and a decrease in the number of peaks called at low cell numbers. In practice, this is what we see. For example, we observed that the amount of chromatin captured for H3K27me3 and RNA Pol II was ~20x higher when purifying from 5×10^7 cells compared to 5×10^4 cells (Supplemental Figure 8). We report the chromatin yield for each protein across the cell titration experiment in Supplemental Table 1 and peak numbers in Figure S9.

Because reduced chromatin yield appears to be the primary limitation at lower cell inputs, we do not believe that decreasing antibody levels would improve this.

In the revised manuscript, we clarified that we used the same bead pool for all cell numbers (Methods, page 81), explain the reasoning behind the number of beads and amount of antibody used (Supplemental Note 2, pgs. 60-63), and describe how the sensitivity is reduced with minimal impact on specificity across cell numbers (Supplemental Note 2, pgs. 60-63).

The authors should comment on whether the approach is robust to the amount of antibody used in addition to the titrated bead pooling detailed in the methods.

To test robustness across a range of antibody amounts, we performed three ChIP-DIP experiments across a 10-fold antibody concentration range (0.1X, 0.5X or 1X, corresponding to 0.15ng, 0.75ng, 1.5ng antibody) with the same amount of lysate. For most antibodies, we see a linear increase in the amount of chromatin (“yield”) with increasing antibody amount across the range. This indicates that the total chromatin capture for each antibody can be titrated by adjusting the antibody amount used and validates the titrated bead pooling approach. Furthermore, we confirmed that the read enrichment over known peak sites for each antibody remains largely unchanged across the three different amounts of antibody. These results indicate that the enrichment measured is generally robust to the precise amount of antibody used within this range.

In the revised manuscript, we describe this titration experiment (Supplemental Note 2, pg 60-63) and present the yield and enrichment comparisons (Supplemental Figure 3).

6. Comparison of signal levels across antibodies targeting different domains can be confounded by the fact that different antibodies have distinct levels of sensitivity (i.e. using the same peak caller, antibody A could have 2-fold greater number of peaks genome-wide than antibody B). Instead of this density plot in Figure 5D, the authors could split peak lists into quintiles or deciles per antibody and determine the extent to those quintiles or deciles correlate across RNAPI, II, and III-bound loci.

We thank the reviewer for raising this point and we apologize for the lack of clarity in our initial explanation.

We agree with the reviewer that different antibodies have intrinsically different sensitivities that confound direct comparisons of signal level. However, Figure 5D is not comparing different antibodies, rather the relative signals of the same antibodies within the same sample across distinct genomic regions. Specifically, the genomic regions evaluated were known promoters of RNAP I, II and III, not regions identified by peak calling.

We have updated the main text (pgs. 12-13) and figure legend (now Figure 6C, pg. 30-31) to clarify how these comparisons were generated and to highlight that these are comparing relative signals within the same samples across genomic regions.

7. In Figure 5A, the authors find certain sets of H3K4me3-marked regions that co-occur with marks associated with active transcription (Set 2) or with heterochromatin (Set 3). However, this does not demonstrate that this association is directly related to “gene type and activity level”. The authors could demonstrate that such transcripts at the RNA-seq level are actually distinct from the remainder of the H3K4me3-marked genes to improve this analysis.

We thank the reviewer for this suggestion. To estimate transcriptional levels at H3K4me3-marked regions we computed the density of RNAPII (which is strongly correlated with RNA transcription level measured by Pro-Seq) within each set and observe a clear difference in Pol II levels and, thus nascent transcription rates, between the different sets.

We now include this data in Supplemental Figure 18.

Minor comments:

1. The authors argue that chromatin state dictates which loci in the genome can be bound by TFs, RNAPII, and other factors (in final sentences of introductory paragraph). However, many features of chromatin structure (e.g. accessibility) are instead patterned by the binding of such proteins. The authors could improve this section by discussing the possibility that the binding of various TFs and enzymatic factors are what drives accessibility patterns in the genome.

We thank the reviewer for this suggestion and have updated the introduction to include this point (pg. 3)

2. For genomic tracks that display binding profiles for the same TF (e.g. Figure 2D), the authors should scale them such that they all depth normalized in the same manner. With these different scales (as indicated by the numbers in square brackets on the right), it is difficult to know if the CTCF antibodies are quantitatively detecting the same level of binding per peak in each condition.

We thank the reviewer for this suggestion and have updated Figures 2C, D, G, H and S6A-C with depth normalized tracks

Reviewer #2

Perez/Gorozny et al describe ChIP-DIP, a new approach for multiplexed chromatin immunoprecipitation in which many epitopes can be profiled by ChIP in a single experiment. A straightforward split-pool indexing strategy is used to deconvolute the chromatin maps. This method represents a significant leap forward in the realm of multiplexed chromatin mapping strategies, which largely are based on CUT&Tag, because it allows profiling of proteins not well suited to CUT&Tag strategies, like many transcription factors, where CUT&Tag approaches often perform poorly. In addition, the approach allows simultaneous profiling of an unprecedented number of chromatin epitopes. The authors use this approach to identify differences in enhancer and promoters that function in different biological contexts. The method and data are of high quality and the manuscript is well written. There are a few outstanding questions and areas for possible improvement detailed below. There are a few important points made in figures or supplement that need to be discussed in the main text. Some of the ‘sanity check’ analyses described early in the manuscript are largely confined to histone modifications. However, given the utility of this approach for transcription factor profiling, a similar set of analyses for TFs is warranted. Finally, a deeper exploration of read depth required at different levels of multiplexing would be helpful for future implementation of the approach.

We thank the reviewer for their thorough review and positive comments. We address each of these specific questions in detail below.

Specific Comments:

1. The experiments testing index cross-contamination in Supplemental Figure 1 are critical for demonstrating the fidelity of ChIP-DIP, but are not meaningfully discussed in the main text. These merit some discussion in the main text and a clearer description in the Supplemental Figure or Legend. (The experiments are well explained in the Supplemental Note, but this could be missed by some readers.)

We thank the reviewer for this suggestion, we now include a more thorough description of these control experiments in the Results section (pgs. 5-6). We have also updated the legend for Supplemental Figure 1 to provide more detail.

2. My expectation is that read depth would need to scale in proportion to the number of antibodies utilized in an experiment, but it is not clear whether this was tested by the authors. This point could presumably be addressed by down-sampling deeply sequenced datasets derived from different levels of multiplexing and assessing the quality of individual maps at each read depth (peak number, correlation with benchmarking datasets, etc).

We thank the reviewer for this suggestion. To explore this question, we downsampled each target from the ChIP-DIP experiments shown in Figure 2A to have the same number of reads across different levels of multiplexing and assessed the quality. We observe strong genome wide Pearson correlations coefficients between downsampled targets, with no trend between strength of correlation and degree of multiplexing. Furthermore, we do not find that the

number of peaks called is influenced by the level of multiplexing when normalizing for read number.

These results indicate that while the total sequencing depth needs to scale with the number of antibodies used, the sequencing depth for individual targets does not depend on the level of multiplexing.

We have included these results in Supplemental Figures 5A-C and discuss these findings in Supplemental Note 2 (pgs. 60-63).

3. The authors perform a series of comparisons between ChIP-DIP and ENCODE datasets for mostly histone modification data in Fig. 1B-E. Since one major advantage of ChIP-DIP over other approaches is its utility for multiplexed TF mapping, it would be very useful to see similar analyses for some TFs, where high quality published ChIP datasets are available.

We appreciate the reviewer's suggestion to further characterize our TF datasets. For TFs where validated ChIP-seq datasets generated by ENCODE are available, we computed the genome-wide correlations and overlap between peak regions measured by ChIP-DIP and ENCODE. In all cases, we observed that they are highly comparable. We have included these comparisons in Supplemental Table 2.

For our mESC TFs, the "reference" map is less obvious because the quality varies widely for existing datasets, making it difficult to determine if differences reflect a poor reference sample or mismatch.

Nonetheless, we appreciate the value in validating these TFs. To do this, we have computed the peak overlap for individual TFs curated from public sources by Remap2022 (a database of transcriptional regulators peaks derived from curated ChIP-seq, ChIP-exo, DAP-se). To ensure only high-quality datasets are included, ReMap2022 implements four different ENCODE-defined quality metrics. We find peaks of ChIP-DIP mapped TFs (FRIP score > 0.5%) are highly enriched for known binding sites, with a median of >90% co-occurrence with reference peaks for both mESC and K562.

In the revised manuscript, we describe these comparisons in the main text (pg. 9) and present the details in Supplemental Table 4.

4. The authors write "For example, we found that RNAP I and II promoters display stronger H3K56Ac enrichment and RNAP I and III display stronger H3K4me2 enrichment relative to H3K4me3 (Figure 5D)." Although these patterns are interesting, it can be difficult to form conclusions about the relative levels of histone marks measured by ChIP, which necessarily uses different antibodies. The maps are normalized for read coverage, but may reflect very different absolute abundances of these marks at individual promoters, due to differences in ChIP efficiency, etc. I suggest modification of the text to reflect the inherent limitations of these comparisons.

We thank the reviewer for raising this point and we apologize for the lack of clarity in our initial explanation.

We agree with the reviewer that ChIP signal is not a direct readout of absolute abundance and that different antibodies have intrinsically different sensitivities that confound direct comparisons of signal level. However, in this context, we are not comparing different antibodies but rather the relative signals of the same antibodies within the same sample across distinct genomic regions.

We have updated the main text (pgs. 12-13) and figure legend (now figure 6, pg. 30-31) to clarify how these comparisons were generated and to highlight that these are comparing relative signals within the same samples across genomic regions.

5. Minor: Fig. 1A – the figure should be modified to reflect sheared crosslinked chromatin is used as an input, not crosslinked cells.

We have updated Figure 1A to reflect this.

6. Minor: Fig. 1B – it would be useful to also see an aggregate track for the ENCODE reference data used in the figure. It may or may not look similar to the ChIP-DIP “All Reads” track.

While we appreciate this suggestion, an aggregate ENCODE track is not comparable to a ChIP-DIP “All reads” track. The “All reads” track represents the combined sample prior to assignment to specific protein clusters, a step which is not present in traditional ChIP-Seq. We include this for ChIP-DIP to illustrate the deconvolution and target assignment.

While we could aggregate the ENCODE tracks, the result would simply reflect the different sequencing depths of those particular experiments and not some underlying property of the data. Accordingly, we’d prefer not to include this as we think it would be confusing and distract from our intended point.

7. Minor: In Figure 5B, the authors note differences in the patterns of histone marks near transcription start or end sites of RNA Polymerase I, II, and III genes. Are these differences due to differences in promoter bidirectionality, which is common for Pol II genes? This could be addressed if a suitable nascent transcript sequencing dataset were available.

To check for changes in histone profiles with promoter bidirectionality, we used GRO-seq to map nascent transcription and identify the subset of genes that are unidirectional versus bidirectional. Comparing the unidirectional subset to all RNAP II genes, we found that the pattern of histone marks at the TSS remained unchanged. We have included a sentence in the results (pg. 12) stating that the difference between patterns at different RNAP gene sets is unlikely due to differences in promoter bidirectionality.

Reviewer #3

Summary: Perez et al. describe ChIP Done In Parallel (ChIP-DIP), a method for conducting ChIP-seq chromatin profiling on a large catalog of targets simultaneously from the same cellular material in the same experiment. The linchpin of the technology is a mixture of paramagnetic beads that pair antibodies with antibody-specific sequencing barcodes, which can be individually identified in a complex mixture with several successive rounds of barcoding via combinatorial indexing. In this way, beads harboring antibodies for several different targets can be combined in the same ChIP-seq experiment. The authors demonstrate that ChIP-DIP yields highly accurate ChIP-seq profiles that are of comparable quality to ENCODE ChIP-seq data. They go on to show that over 100 targets can be co-profiled in the same experiment. By analyzing co-occurring patterns within these data, the authors are further able to characterize distinct chromatin states at initiation sites for RNA polymerase I, II and III, and distinguish apparent gene and enhancer regulatory subtypes with unique combinations of histone acetylation. In all, the authors demonstrate a compelling technological adaptation that promises to make “consortium-scale” ChIP-seq possible in a single lab.

Evaluation: This is a clever method, and the controls for data quality appear to be thoughtful, careful, and well conducted. However, there are more subtle sources of potential background that might be worth addressing, including cryptic multi-bead interactions. I also think there are a couple technological clarifications that should be made for the benefit of potential users of ChIP-DIP: First, how the yield and data quality compare with “real” input cell numbers, and second, how ChIP-DIP compares with alternative techniques such as CUT&RUN and CUT&Tag, for which high-throughput, “low-touch” approaches are already available.

Impact: I agree with the authors that this technology, if sufficiently robust and controlled for, offers the potential for “consortium scale” ChIP-seq in a single or a few experiments. Such an accomplishment would democratize large-scale ChIP-seq in a way that hasn’t really been possible previously. Although it is worth weighing its utility against non-ChIP-seq alternatives that have accomplished some of the same goals, this work should still be of broad interest to the chromatin and gene regulation fields.

We thank the reviewer for their positive comments and detailed review. We address each of these specific questions below.

Major Comments:

- One potential source of background the authors have not explicitly considered here is the binding of a single particle by two beads. This could be a particularly insidious source of background because it may manifest in multivalent “stickiness” where one or a few “true” co-modified particles that gets bound by two different beads could seed the association of many other particles that wouldn’t otherwise associate. Although the correlations shown throughout this manuscript show that there is good specificity of targets on a genome-wide level, one could imagine this phenomenon subtly degrading the specificity at the locus level in a way that would be difficult to detect but could impact downstream analyses. A few points here:

We thank the reviewer for raising this important point. Indeed, this was a major concern we shared when we started developing this method. However, we find that, in practice, this happens very rarely.

Specifically, if a single particle of chromatin were bound by two or more beads, we would detect a single cluster with two or more types of oligos, one from each bead-type. To explore this issue, we measured how often we observe more than one oligo-type within a cluster and found that in virtually all cases (96%), chromatin clusters contained only a single oligo-type. This was true even when focusing only on clusters where we measured >5 bead-oligos per cluster, indicating that this uniqueness represents specificity of assignment and not simply lack of distinct oligo detection. In the rare instances when we do observe beads without dominant oligo type (maximum representation oligo represents < 80%), we exclude such ‘clusters’ from our analysis.

We present this analysis in Supplemental Figure 1B and describe these observations in more detail in the Main text on pgs. 5-6.

o The concern here is partly addressed in the human-mouse mixing experiment, but it’s hard to know to what degree “bead stickiness” is contributing since this phenomenon would be indistinguishable from a violation of the “birthday problem” in which two distinct particles with different antibodies get the same barcode combination, and the authors haven’t articulated what the expected mixing rate is here based on the number of input particles vs. possible barcode combinations. Can the authors comment on the degree to which birthday problem effects vs. bead stickiness effects are at play here? If the authors were to reduce the input relative to the possible barcodes, would mixing rate go down (indicating a birthday problem problem) or would it stay roughly the same (indicating a bead stickiness problem)?

This is an important question that gets at the heart of the specificity that is achievable by split-pool barcoding methods.

Briefly, we ensure that the probability of a ‘collision’ (two distinct particles get the same barcode by chance akin to the ‘birthday problem’) is extremely low because the total number of barcodes is in vast excess (>10X) to the maximum number of possible beads in each experiment. To accomplish this, we measure the number of beads used (b, which is the maximum number of assignable bead particles) and select the number of split-pool tags (t) and rounds (r) such that $t^r \gg 10 \times b$.

For example, in a typical experiment with ~10 million beads, we perform 7 rounds of split-pool with 24 barcodes per round, resulting in ~7 billion unique barcodes. In this condition the probability of a ‘collision’ is <0.0007 of all beads. In other words, 99.93% of all beads would be expected to receive a single unique barcode.

While the reviewer is correct that ‘collisions’ are not 0, this would represent only 7,000 beads that incorrectly share a barcode with one other bead. However, because such collisions are expected to be random, any spurious read-antibody assignments generated by these rare

collisions would represent an increase in the random background measured. Furthermore, such collisions would likely be thrown out because they would be clusters that contain more than one antibody ID oligo and not pass the 80% unique antibody ID oligo threshold.

Because ‘collisions’ do not appear to be a significant concern in our experiments, any additional mixing in the human/mouse experiments likely reflects other aspects of the protocol, including chromatin that dissociates and reassociates to a different bead of the same type (as further described below).

We have added the rationale for selecting the barcode numbers in Supplemental Note 2 (pg. 60-63).

o If observed mixing is 4-6%, actual mixing is expected to be double that due to unobserved intra-species mixing, and so the real mixing rate (8-12%) should be reported, especially since it is actually rather high as compared to many other combinatorial indexing-based single-cell profiling approaches.

We thank the reviewer for raising this important point which we now realize we failed to properly explain in the original manuscript.

Briefly, we performed this human/mouse mixing experiment to determine whether specific chromatin (e.g. DNA bound by H3K4me3) was moving from one bead to another bead of the same type during the ChIP-DIP procedure. This would be a concern because the assignment of protein to DNA is dependent on the bead and DNA sharing the exact same string of tags (barcode), which requires that the DNA remains bound to the same bead throughout the duration of split-and-pool barcoding. Dissociation and recapture would lead to the inability to assign the DNA to a protein because the DNA would contain a barcode that consists of tags linked partially to different beads.

Importantly, such instances would not lead to false positive assignments but instead would lead to lack of assignment. In other words, this would not impact specificity but would impact yield. Nonetheless, if this occurred frequently then the ChIP-DIP approach would not work because we would be unable to accurately assign proteins to DNA.

Accordingly, we wanted to test how often this might occur. To do this, we used a human/mouse mixing experiment where each species was captured onto their own beads containing the same antibody and then we measured the frequency of dissociation and reassociation events based on the inter-species assignment frequency. Indeed, the reviewer is correct that the frequency is higher than the observed 4-6% because of the unobservable portion of human/human and mouse/mouse events. However, the fact that overwhelming majority (>90%) of DNA is uniquely resolvable. While this quantification indicated that this was not likely to be a problem for the method, we have confirmed that in practice this is not a problem as we have been able to successfully resolve the protein to DNA mapping of hundreds of distinct proteins.

We have reworked the explanation in the main text to clarify the motivation and interpretation of these experiments and to more accurately state the observed mixing rate (4-6%) likely reflects a higher underlying mixing rate of 8-12% and the interpretation that the vast majority of clusters (>88-92%) remain intact throughout the ChIP-DIP procedure (pgs. 5-6).

o mESCs might be expected to suffer from bead stickiness more acutely since bona-fide H3K27me3-H3K4me3 bivalent domains might seed apparent H3K27me3-H3K4me3 co-occurrence that doesn't actually exist.

We appreciate this concern, however, in practice we do not see this. Briefly, if multiple beads (H3K27me3 and H3K4me3) were bound to the same chromatin fragment, we would detect individual clusters that contain oligos corresponding to H3K27me3 and H3K4me3. We rarely see this, with <0.13% of all H3K4me3 or H3K27me3 clusters containing both. This indicates that we do not have a significant degree of “bead stickiness” even when multiple beads could be seeded by *bona fide* chromatin sites.

Can the authors show explicit comparisons, both genome-wide and on the locus level, between mESC H3K27me3 and H3K4me3 ENCODE ChIP-seq and ChIP-DIP to demonstrate this isn't the case? Both genome-wide correlation heatmaps and locus-specific browser shots for bona fide bivalent and non-bivalent regions as defined by ENCODE ChIP-seq would seem appropriate here.

We thank the reviewer for the suggestion. We have now added a supplemental figure demonstrating genome-wide enrichment of H3K27me3 and H3K4me3 over bivalent and non-bivalent regions (as defined by Mikkelsen 2007) in mouse ES cells. We find that our enrichments are highly comparable to that observed by ChIP-Seq data of these modifications generated by ENCODE. We include this global analysis and locus-specific examples in Supplemental Figure 17.

- Lines 70-72: While I appreciate the potential advance represented by this technology, this particular sentence acts as though time has stood still since ENCODE. There has been a decade of technological improvements since then, including CUT&RUN and CUT&Tag that are now in widespread use precisely because of their utility across a range of targets and their proficiency on the productivity metrics the authors are trying to address.

We apologize that our choice of wording implied that ‘time has stood still’ or otherwise inadvertently discounted important advances in the field. This was certainly not our intention and we have reworked this sentence to make this clear (pgs. 3-4).

According to the 2018 Janssens et al. paper cited here (#19), high-throughput automated CUT&RUN can be implemented for \$75/sample in two days per 96 samples. If we're substantially less generous and call it \$150/sample in 4 days, to recreate the work listed in Supplementary Table

1 (614 samples by my count) would cost \$92,100 and take ~1.5 months in a single lab. How does ChIP-DIP compare to these? Given that the authors cite cost, manpower, and time as important factors upon which ChIP-DIP improves, it would make sense to compare exact numbers for those variables with this and/or other up-to-date alternatives so that potential users can make an informed decision about the comparative advantages of this approach.

Because pricing of all assays depend on many factors - including but not limited to local reagent costs, personnel costs, inflation - we do not believe that a direct cost comparison to other methods makes sense. The same is true for time and manpower, because a lab that invested in a liquid handling robot would require less time and fewer people to perform ‘high-throughput automated’ CUT&RUN or ChIP-Seq than a lab without it. Such liquid-handling robots are not standard in most labs and the pricing and timing estimates do not factor in the high equipment, maintenance, and reagent costs associated with liquid-handling automation of this procedure.

Nonetheless, our goal was not to criticize other possible approaches, and certainly in some specific labs with specialized training and equipment, other approaches might certainly be reasonable options. Rather, our goal was to highlight the benefits of ChIP-DIP and why it will be a useful approach for many labs specifically because ChIP-DIP enables highly multiplexed mapping of hundreds of proteins within a single experiment without the need for any specialized equipment or training.

- Lines 135-140: I think it’s worth considering the number of effective cells that are actually being profiled here. It is well established that combinatorial indexing-based methods for single-cell molecular profiling experience significant cellular loss relative to input. This is owing to both the loss of cells/DNA molecules through buffer exchanges and molecular processing steps, and the fact that only a very small proportion of the possible barcode combinations available end up being used. Therefore, it seems as though the “cells/target” calculated for Fig. 2E is likely optimistic, and the user can’t necessarily be confident that the profiles they recover are truly equivalent in quality and library complexity to what would have been obtained from profiling the equivalent number of cells in isolation via ChIP or CUT&RUN/Tag.

We apologize for any confusion caused by our “cells/target” comment. We agree with the reviewer that, due to cellular losses during workup, the final number of cell equivalent is lower than the starting number of cells and our “cells/target” would represent an overestimation. We also agree that a single ChIP experiment starting with these “cell/target” amounts in many cases, would likely yield worse outcomes or fail because the amount of material is quite low.

Our goal here was not to compare our results to ChIP-Seq measurements at low cell numbers but rather was intended to highlight the fact that, because ChIP-DIP captures chromatin across a diverse pool of targets, fewer total cells are needed compared to mapping many proteins using a one-by-one approach. For example, if one needs 100,000 total cells to map an individual protein then to map 35 individual proteins would require 3.5 million total cells. In contrast, by performing this pooled experiment, we would be able to map 35 proteins

using the same 100,000 total cells. In this context, we used the “cells/target” estimate to indicate the number of cells per target that would currently be needed to generate these datasets using a traditional one-at-a-time approach.

We note that it is currently not possible to generate these same dataset using ChIP-Seq with the same effective per-protein starting material amounts because current ChIP-seq variations designed for low cell input (e.g. Nano-ChIP-seq, LinDA, ULI-NChIP) still require several thousand cells (>5,000 cells for histone modifications, >10,000 cells for abundant chromatin binding proteins such as CTCF, and > 50,000 for transcription factors such as TBP).

We have rephrased the section of the text to clarify that we are referring to the total amount of cell equivalents needed using individual assays (pg. 7).

Could the authors provide some demonstration of the quality (e.g. FRIP) and estimated library size (e.g. via PicardTools) of their ChIP-DIP profiles that they can benchmark to a range of ChIP profiles from different numbers of cells so that a more accurate estimate of effective cells profiled could be ascertained? This could probably be accomplished without extra experiments by simply downsampling a single individual ChIP-seq experiment to represent different effective cells profiled and comparing it to ChIP-DIP.

While we appreciate the suggestion to downsample read depth, this alone does not accurately reflect the issues associated with mapping chromatin in lower cell numbers. For example, a major issue for mapping using low cell inputs is the high-level of background that is captured as well as the baseline loss of material due to retention on plastics (e.g. tubes, tips, etc) that have larger impacts when material is limited. This is why it is often a challenge in practice to perform genomic assays in small numbers of cells.

Nonetheless, we appreciate the suggestion to demonstrate the quality of ChIP-DIP profiles across the range of cell numbers. Because traditional ChIP-seq libraries are not commonly available in the cell number range of this experiment (<1M), we compared our ChIP-DIP data to publicly available low cell input CUT&TAG libraries for the targets that are available.

To estimate complexity, we computed “complexity curves” (reads sequenced vs non-duplicate reads) for each individual target. As expected, lower cell number results in a stepwise downward shift in the complexity curves. For many targets, we find that ChIP-DIP is highly comparable – and for some targets vastly outperforms – the complexity measured for CUT&TAG at equal or higher cell numbers. For example, for CTCF, ChIP-DIP at 5M cells is similar to CUT&TAG performed with 10M cells and ChIP-DIP with 50K cells has significantly higher complexity (as evidence by the upward shifted curve) than CUT&TAG at 500K or 100K. These results are described on pg. 7 and presented in Supplemental Figure 8.

We also computed FRIP scores as a function of number of peaks for ChIP-DIP and CUT&TAG. We found that for histone modifications with narrow profiles (H3K4me, H3K27ac) as well as CTCF, the FRIP scores for ChIP-DIP decreased minimally as cell numbers decreased and were comparable or exceeded the FRIP scores for CUT&Tag. While the number of peaks decreases for most targets with decreasing cell number, virtually all of the peaks identified at low cell numbers are identified at higher cell numbers, indicating that reduction in peak numbers and reduced FRIP scores are due to a loss in sensitivity, but not specificity.

These results are described on page 7 and in Supplemental Note 2 (pgs. 60-63) and presented in Supplemental Figures 8-9.

- Lines 58-60: Just to note that there are actually quite a few methods for multifactorial chromatin profiling that have been developed in the past few years. In addition to the ones cited here, there are also MulTI-Tag (Meers et al. Nat Biotech. 2022), NTT-seq (Stuart et al. Nat Biotech. 2022), and uCoTarget (Xiong et al. Sci Adv. 2024, and on bioRxiv since April 2023). However, all of these technologies also have the benefit of detecting bona-fide co-occurrence of histone modifications in the same single cells, which ChIP-DIP does not as presented here, so the comparison is somewhat apples and oranges. The more appropriate comparison for ChIP-DIP would probably be with high-content ChIP or CUT&RUN/Tag approaches (as outlined above).

We thank the reviewer for these suggestions, we have added these additional references in the Introduction and have added a conceptual comparison of ChIP-DIP to these other high-throughput methods in the Discussion (pgs. 3, 16)

- Figure 4: Accurate transcription factor profiling has always been challenging and the array of TFs profiled here is impressive, but there should be quality metrics reported for every transcription factor included here, for instance the rank of the TF's expected binding motif in the top recovered motifs, and the percentage of fragments containing an expected binding motif.

We appreciate the reviewer's suggestion to further characterize our TF data. We now provide a comprehensive analysis of motif prediction (Supplemental Table 5). Briefly, we focused on the TFs that had a sufficient number of peaks needed for *de novo* motif discovery. We found that for ~80% of these TFs, we identified a *de novo* motif ranked within the top two that matched the expected motif, TF family member motif, or frequently co-occurring motif (e.g. other promoter TF promoter-associated TF). For the majority of the remaining TFs, the expected motif was detected with a significant *p*-value within the top ten ranked motifs. This demonstrates the high-quality TF profiling by ChIP-DIP.

We describe these results on page 9 and present these data in Supplemental Figure 13 and in Supplemental Table 5.

- How is the ChIP-DIP target-specific yield dependent on the other species added to the pool? I'm

particularly concerned that highly similar epitopes, e.g. different methyl valency states of the same modification, might promiscuously bind to each-others' antibodies when they are both in the mix, especially since it's known that many histone modification antibodies can have low specificity (for instance, as catalogued here: <https://chromatinantibodies.com/>). Have the authors evaluated how correlations change for the same ChIP-DIP target when it's profiled in concert with different partner targets in the same pool?

We agree with the reviewer that the question of possible pool composition effects is very important and a question that we too were concerned about when designing this method.

To explore this, we performed ChIP-DIP for the same set of proteins in different pool numbers and containing different antibody compositions. For example, we mapped H3K4me3 in a small pool without overlapping targets as well as in a larger pool containing antibodies that target all three methylation states of H3K4 (H3K4me1, H3K4me2, and H3K4me3). In all cases, we found that the observed tracks (e.g. H3K4me3) remain highly correlated when measured in all pools, including when directly comparing pools with (35 pool) or without (10 pool) antibodies to highly similar epitopes. We performed similar analysis on multiple proteins within the same complex (e.g. EZH2, EED, and SUZ12 components of the PRC2 complex) and for different post-translational modifications of the same protein (e.g. various phosphorylation states of RNAP II). In all of these cases, we are able to generate high-quality binding maps for each of these proteins regardless of the pool composition.

The most extreme example of this is when using two antibodies targeting the same protein within the same pool. Indeed, we found that mapping CTCF within a pool containing a single antibody or in other pools containing two distinct CTCF antibodies showed highly comparable results and the two distinct CTCF antibodies within the same sample both led to highly comparable profiles.

We present these experiments and analyses in Figure 2 and Supplemental Figure 5 and we present a complete list of pool sizes and compositions in Supplemental table 1.

- Figure S7: Histone acetyl antibodies in particular are known to be highly promiscuous. Do the co-modified clusters you identify here line up with what has been found from mass spectrometry studies of co-modified histone tails (to the extent that they exist)?

We appreciate the reviewer's suggestion to investigate co-modified histone tails. Of note, the states described in Figure 8 consist largely of modifications that occur on distinct histone tails (e.g. H3K27ac and H2BK20ac), not co-modifications on the same histone tail and such cross-histone co-modifications are rarely measured by mass spectrometry for technical reasons.

Nonetheless, we explored co-modifications on the same histone tail using CrosstalkDB (<https://protprotocols.bmb.sdu.dk/crosstalkdb.interface/faces/showHistone.xhtml>). While specific examples of co-occurring histone acetylation patterns have been identified

(H3K14acK18acK23acK27ac), they do not correspond to the modifications profiled in our experiment.