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Nat Aging. Author manuscript; available in PMC 2024 February 01.

Published in final edited form as:
Nat Aging. 2023 August ; 3(8): 921–930. doi:10.1038/s43587-023-00448-4.

Considerations for reproducible omics in aging research

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Abstract

Technical advancements over the past 2 decades have enabled the measurement of the panoply of molecules of cells and tissues including transcriptomes, epigenomes, metabolomes, and proteomes at an unprecedented resolution. Unbiased profiling of these molecular landscapes in the context of aging can reveal important details about mechanisms underlying age-related functional decline and age-related diseases. However, the high-throughput nature of these experiments creates unique analytical and design demands for robustness and reproducibility. In addition, 'omic' experiments are generally onerous, making it crucial to effectively design them to eliminate as many spurious sources of variation as possible, as well as account for any biological or technical parameter that may influence such measures. In this manuscript, we provide general guidelines on best practices in the design and analysis of 'omic' experiments in aging research, from experimental design to data analysis and considerations for long-term reproducibility and validations of such studies.

Introduction

With the democratization of high-throughput 'omics' experiments, it is now easier than ever to get genome-wide information about the molecular pathways governing how cells, tissues

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Author Contributions
P.P.S. and B.A.B. conceived and wrote the manuscript together.

Competing interests
The authors declare no competing interests.

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and organisms age. Indeed, over the past decades, we have gained the ability to generate robust high-throughput measures describing the molecular landscapes of biological systems at multiple levels (*e.g.* genome, epigenome, transcriptome, metabolome, proteome). Such high-level analyses enable unbiased analyses of how these systems respond to complex biological contexts (*e.g.* aging), and enable researchers to generate new hypotheses about the molecular networks that regulate such processes. Their high-throughput nature creates unique analytical and design demands. For example, the ideal number of replicates in low-throughput experiments can be statistically determined using power analyses or simulations, whereas every molecule in an 'omic' experiment will have its own effect size and variance in response to biological cues, which cannot be reasonably determined *a priori*. In addition, because of the cost involved, it is critical to effectively design such experiments to account for biological or technical parameters that may influence measures of cellular 'omes' with aging. This is important not. only to improve reproducibility and interpretability of experiments, but also to determine how general the conclusions that can be derived from an 'omic' experiment are to explain aging (*i.e.* only relevant to one sex/strain, or multiple).

Although the most mature and robust 'omics' technologies usually interrogate populations of cells (*i.e.* bulk 'omics'), recent advances now allow such investigation to occur at the level of single cells or single nuclei. Single cell profiling technology theoretically has the unique power to detect underlying cell population heterogeneity. Indeed, single cell 'omics' can be very useful in the identification of the cellular composition of a population or tissue of interest, although their power is more limited for identification of, for example, differentially expressed genes in a cell type of interest, due to sampling bias, relatively high technical noise and an illusion of large numbers (*e.g.* dropouts[1], high false discovery[2]). Thus, special considerations should be taken when designing and analyzing such experiments in aging research and beyond.

Aging research has rightfully embraced the "omics" revolution, which has already enabled many important discoveries about the aging process. Unbiased high-throughput experiments in aging animals has already provided important insights about aging: discovery of widespread immune activation across tissues[3, 4, 5], development biomarkers and clocks to quantify aging using machine-learning approaches[6, 7], and identification of determinants of species longevity[8, 9, 10]. Due to laborious and time consuming nature of aging experiments, effective design, generation and analysis of "omics" data is especially critical in aging research. General guidelines for "omics" studies have been discussed elsewhere[11, 12, 13, 14, 15, 16, 17]. In this manuscript, we discuss specific guidelines for best practices in the design and analysis of 'omic' experiments in aging research, focusing on biological, technical and analytical considerations, and validation of such "omics" results, with a more particular focus on sequencing-based omics technologies.

1. Biological considerations for 'omic' studies of aging

For any successful aging omics experiment, many key steps must occur very early in the design and sample collection phase. Since any mistake at this stage cannot be compensated for in the downstream analysis, it is crucial to consider sources of biological variation (*e.g.* sex, reproductive status, circadian variation, genetic background, microbiome, *etc.*) and how

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they interact with the studied process (*e.g.* biological aging). Indeed, uncontrolled influence of such sources of biological variation will lead to confounded designs from which no robust interpretable results can be derived. Like any other aging experiment using animals, aging "omics" should adhere to general guidelines for animal use in aging reseach[18] and general reporting guidelines for animal research (ARRIVE)[19]. In addition, we discuss here important sources of biological variation that may be relevant to account for in the design of a robust "omics" experiment for the study of aging (Figure 1; Table 1).

Biological sex.—Accumulating evidence has shown that biological processes in general, and aging in particular, are very sex-dimorphic even outside of reproductive function[20]. Typically, sex-differences can derive from differential gonadal hormone prevalence (*i.e.* estrogens vs. androgens) or differences in sex chromosome complement (*i.e.* XX vs. XY in mammals)[21]. Of specific relevance to omics studies, gene expression and chromatin structure show marked differences throughout life[22, 23], even when focusing only on autosomes. In addition, different age-related 'omic' trajectories can be observed depending on biological sex[24]. For these reasons, aging omics studies should ideally include both female and male samples in sufficient numbers (see below) to detect any potential sex differences in aging trajectories, especially if studies aim at deriving generalizable conclusions for the field. In the case that inclusion of both sexes is not feasible (*e.g.* cost reasons), which sex was used should be explicitly and prominently mentioned, and this choice should be discussed as a caveat. Further, it is never appropriate to: (i) not describe the sex of the samples used, or (ii) use animals of different sexes in different groups (*e.g.* males only for the young group and females only for the old group). In addition, it is also generally discouraged to derive samples pooled from animals of mixed sex, unless there is reasonable biological justification, as there is a concern that effects could average out and provide a misleading picture existing in neither sex separately.

Reproductive status.—In addition to their role in reproduction, sex hormones influence somatic tissues by signaling through broadly expressed dedicated receptors, such as estrogen receptors (*e.g.* Erα, Erβ) or androgen receptor (*i.e.* Ar). Thus, different reproductive states are not neutral with respect to somatic biological processes, including at the "omic" level. Indeed, gene expression and chromatin accessibility can be directly regulated by cyclic estrogen fluctuations during the murine estrus cycle, for example in the brain[25]. Pregnancy itself is accompanied by major hormonal changes and has been shown to impact gene expression in human immune cells[26], although how long these changes persist after parturition is unclear. Further, mating itself (even in the absence of fertilization or pregnancy) alters the rate of aging in female mice[27]. Thus, the long-standing practice of using retired breeder mice as a source of cost-effective aged animals may have unforeseen and uncontrolled impact on omics data. Thus, we recommend prioritizing the use of unmated animals unless the impact of breeding and/or co-housing on aging 'omic' phenotypes is a desired outcome variable.

Circadian effects.—In animals, the circadian system controls daily biological rhythms, with both central regulation and cell autonomous circuits, whose dysregulation is thought to play an important role during aging[28]. Circadian biology is mediated in part by large

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changes in transcriptional regulation that lead to tissue adaptation throughout the day, with the daily regulation of thousands of genes[29]. This impact of circadian rhythms on genomic regulation has several consequences to avoid confounds in omics experiments. First, one should make sure to stagger sample collection across considered groups so that there is no circadian pattern to the collection of each group (*e.g.* in an experiment comparing young and old animals, alternate sampling of old and young animals). Second, the approximate time of day at which animals are euthanized for sample collection should be reported, so that data can be appropriately contextualized in future studies.

Post-mortem interval.—Related to circadian impacts, the post-mortem interval has been found to yield reproducible transcriptional changes in human[30] and mouse[31] tissues. In addition, disease status[32] and storage length[33] can also affect gene expression in post-mortem tissues, especially relevant to human samples (see below). Since this phenomenon is likely to exist across species and tissues, post-mortem interval should be accounted for in 'omic' experimental designs. Thus, we recommend using staggered collection approaches, so as to help mitigate the impact of post-mortem transcriptional remodeling and to avoid confounding the impact of age groups with that of post-mortem transcriptional remodeling.

Genetic background.—The impact of genetics on gene expression patterns in humans has become better appreciated with large scale efforts like GTex[34].Because genetic factors can regulate organismal aging in model organisms, and because response to pro-longevity interventions (including at the 'omic' level) may be influenced by genetic background (*e.g.* calorie restriction in mice[35, 36]), accounting for genetic variability is key for statistical power in 'omics' studies. Since genetic variation may decrease the signal-to-noise ratio of such experiments, note that profiling genetically diverse samples may require higher sample numbers to achieve adequate sensitivity (see below).

Genetic divergence of inbred animal stocks.—Inbred animals have been broadly used in aging research, since their lack of genetic diversity may limit non relevant noise in data and makes the analysis of the role of specific genes more tractable (*e.g.* through knock-out experiments). For instance, labs use mice from C57BL/6 substrains, worms from the N2 strain, African turquoise killifish from the GRZ strain, etc. However, due to reproductive isolation and genetic drift (since mutation rates are not null), such inbred stocks tend to significantly diverge with time[37, 38]. For mice, since they can be sourced from different vendors (*e.g.* Jackson labs, Charles River), this has led to the emergence of vendor-specific substrains (*e.g.* C57BL/6J *vs.* N), that have fixed dozens of genetic variants leading to many phenotypic differences with relevance to aging[37]. Vendors like Jackson labs try to minimize the impact of genetic drift on their animals by regularly rederiving animals from frozen embryos. For animal colonies maintained by labs and not a central provider (*e.g. C. elegans*, killifish), reproductive isolation is expected to lead to genetic drift as each lab maintains their own animal colony, with potential impact on aging phenotypes[38]. Unfortunately, it is not always possible to source all animals over multi-year studies from the same vendor (*e.g.* mice), or to control for mutation accumulation over repeated generations of breeding in a lab-maintained colony (*e.g.* worms, killifish). Thus, in the context of omics studies, it is recommended to ensure that all samples to be compared are derived from the

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same substrains (and/or within a similar timeframe), or, if unavoidable, that all groups to be profiled contain representation from all different isolates.

"Supplier" effects and the microbiome.—Housing conditions across different animal facilities can vary widely (*e.g.* chow composition, temperature, ambient noise, etc.). Indeed, even with dedicated standardization efforts, the NIA intervention testing program has reported differences in basal longevity and response to interventions in genetically controlled UM-HET3 mice[39]. A potential driver for such differences is the microbiome, an emerging factor in aging, longevity and age-related diseases[40]. Importantly, microbiome composition can vary widely across mouse suppliers[41]. In controlled microbial transplant experiments, compositional differences in healthy microbiome can significantly influence metabolism, behavior, cognition, blood cell counts[41] – and, although this has not been assayed yet, there is no reason to believe that there would not be concomitant 'omics' changes as well. Thus, similar as above, it is recommended to make sure that all samples to be compared are derived from animals obtained from the same vendor and within a reasonable timeframe, or, if unavoidable, that all groups contain animals from all sources (*i.e.* young animals cannot be compared to old animals if sourced from a different supplier, even if they come from the same genetic stock).

Pathological findings and censorship.—There is much debate on whether studying aging should include only "healthy" aging examples, or whether the spectrum of "unhealthy" aging should also be represented. Significant numbers of laboratory mice past middle-age will present with some age-related pathology (neoplastic or non-neoplastic)[42]. Although this has not necessarily been studied quite as closely, it is expected to occur in other commonly used models in aging research as well. Although the choice to only include macroscopically "healthy" animals may make sense for some study designs, we believe that this decision should be made explicit, and criteria for animal censorship should be transparent. Such reporting is crucial so that the scientific community can interpret a dataset as representative of "normal" *vs.* "healthy" aging.

Choice of age groups in animal models.—Choosing age groups for "omics" profiling will have large impact on the results. The simplest design for aging "omics" experiments using model organisms will include a "young" group compared to an "old" group. In general, young animals should be past sexual maturity, ideally when reproductive processes are stabilized[18]. In contrast, old animals ideally correspond to advanced ages before population crash to avoid the so called "survivorship bias"[18]. To note, ages representative of young *vs.* old states will differ as a function of species, but also of system of study (*e.g.* reproductive *vs.* somatic aging occur on different time scales in mammalian females). Inclusion of additional intermediate time points may reveal non-linear dynamic regulation of molecular networks, and reveal differential age-related dynamics in abundance changes of transcripts, proteins or metabolites (*e.g.* transcripts or small RNAs[43], proteins[44]). To note, although informative, adding additional time points may not always be feasible based on animal availability and costs. In addition, multi-time point designs will also require more complex analytical tools. Thus, we encourage the community to consider the inclusion of ages across the lifespan when feasible.

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Considerations for human samples.—The considerations discussed above are also broadly applicable to human 'omics' datasets (*e.g.* the need to account for sex/gender, reproductive status, circadian effects, etc.). However, unlike most animal models, humans have greater genetic diversity and heterogeneity. Indeed, human 'omic' data can be influenced by behavioral factors (*e.g.* dietary preferences[45], etc.) or even the socio-economic status[46] of individuals. Because these factors are not always easy to account for using biobanked samples or even during study recruitment, this makes aging 'omics' studies using human samples uniquely challenging to identify consistent age-related patterns. If human samples are obtained from biobanked post-mortem tissues, sample integrity and quality must be insured by evaluating the purity, quantity, concentration and integrity of the samples. In addition, it is key to account for post-mortem interval when selecting samples from biobanks to account for its effect on 'omics' profiles[30] (see above). Importantly, humans are generally more genetically heterogenous than animal models, with human genetic diversity having been shown to impact gene expression[34], chromatin binding[47], chromatin accessibility[48], DNA methylation[49] or proteome[50] landscapes. Importantly, detecting significant transcriptional changes with age in the GTEx RNA-seq data required accounting for genetic variability using the top 3 principle components of the genotype of donors[51], highlighting the need to account for genetic variation in human 'omic' studies. Greater genetic diversity often leads to significant variation in gene expression and regulation between individuals[52]. Therefore, extra care is needed in experimental design using human samples to account for factors, including population diversity, ethnicity, gender/sex, socioeconomic status and comorbidities. Because of these unique challenges, specialized analytical tools may be needed to account for unique patterns of genetic or environmentally-driven heterogeneity across human samples[53, 54] to gain meaningful insights from 'omics' aging studies. Finally, it is critical to ensure that appropriate ethical clearance and informed consent has been obtained for the collection and use of the human tissues/samples, and that any data generated is handled, stored and shared in accordance with relevant data privacy regulations[55, 56].

2. Technical considerations for 'omic' studies of aging

Although the design and analysis of aging 'omic' studies shares many technical considerations with that of other biological contexts, there are also important considerations that will be more specifically relevant in the context of aging studies. Here, we discuss and highlight both more general and more specific technical considerations for successful, reproducible, and robust analysis of 'omic' changes with aging (Figure 1).

Defining biological vs. technical replicates.—Biological replicates are central in any 'omics' experiments to assess the reproducibly of observed changes and identify features that are consistently different between young *vs.* old animals. Biological replicates are samples that are taken from different animals (or different pools of animals) within a population. Samples derived from the same individual (e.g. coming from any technical variation in sample processing, derived from separate fragments of the same tissue, etc.) constitute technical replicates. When pooling multiple replicates to increase the quantity of input in a bulk 'omics' experiment (which may be needed in some instances *e.g.* if sample quantities are inadequate to produce enough material from a single animal), a pooled

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sample is still considered as a single biological replicate, and multiple 'pooled' samples should be included in the design. While technical replicates may be important to identify non-biological sources of experimental variability, as a rule of thumb, a minimum of three biological replicates should be included for each group in bulk omics experiments (e.g. RNA-seq), to minimize the odds of an outlier sample disproportionally driving results. The reliability of differential expression analysis improves with increasing numbers of biological replicates, especially for genes with lower fold change[57, 58, 59]. Use of lower numbers of replicates does not increase the false positive rate (*i.e.* features incorrectly labelled to be differentially expressed), but reduces power to detect truly differentially regulated genes[57, 58, 59]. Thus, based on benchmarking studies, 'omic' experiments with 4–6 biological replicates are likely to capture key salient changes between the conditions of interest (*i.e.* 60–90% of changes detected with larger replicate number[57, 58, 59]), although they may miss more subtle changes (*e.g.* higher rates of false negatives in low sample conditions). To note, based on sample availability and/or budget constraints, smaller number of replicates per time point may be considered for aging time-series experiments given that there is sufficient depth in time-series sampling[60]. In general, emerging approaches for statistical power analysis may be used to identify the replicates and sample size for differential expression studies, provided that some adequate pilot 'omic' data exists[61, 62]. However, an important caveat to consider for any power calculation in 'omics' is that each gene, feature, or region has its own effect size (*i.e.* fold change in expression or accessibility) and variance (*i.e.* level variability between samples), and thus its own power threshold. If required due to higher sample variability, it is possible to add new samples in separate batches in follow up experiments after a pilot, as long as all biological groups are represented across subsequent batches (see below). However, it is important to note that adding new samples after a pilot experiment in 'omics' will lead to issues related to batch effects (see below), so using a reasonable number of replicates (4–6 per group) *a priori* should be preferred to maximize biological signal, and avoid the need for batch correction.

Replicates and age-related stochasticity.—Specific to aging biology, aging is thought to harbor a strong stochastic component and different animals may age along different trajectories with distinct molecular changes[63]. For example, aging has been associated with increased gene expression and epigenetic noise, with higher inter-individual variability observed in old animals[64, 65, 66]. To note, a recent meta-analysis suggests that age-related transcriptional noise may be driven by changes in cell composition of tissues[67]. Thus, it is possible that additional biological replicates may be required when including older animals to detect robust aging signatures despite increased variability. Specific benchmarking studies including large number of biological replicates in young *vs.* old samples will have to be conducted to determine whether age-related stochasticity warrants increased numbers of replicates. However, one should be careful with proactively using increased numbers of replicates for old vs. young animals, as sample number imbalance itself can greatly impact the results of differential gene expression analyses[68]. The importance of not over-concluding from relative lack of changes in low sample studies related to aging cannot be overstate. Indeed, although early studies of aging proteomics failed to detect robust age-related changes[69], later more powered studies were able to detect robust differences[44]. Higher

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numbers of replicates are particularly important for studies involving human subjects with high heterogeneity and diversity among individuals.

Replication in single-cell aging 'omics'.—Similar to bulk 'omics', biological replicates in single cell experiments should be derived from cells coming from distinct individuals. Because single-cell experiments are considerably more expensive than bulk 'omic' experiments, there are economic constraints to consider when performing multiple sets of independent experiments coming from different animals. This may be especially the case in the context of aging where many rare cell types may no longer be sufficiently abundant in old animals (*e.g.* neural stem cells[70]). A key strategy to save cost without compromising quality can be to multiplex biological replicates at earlier stages of library preparation (while allowing sample-level cell tracking[71, 72, 73]), with downstream computational de-multiplexing to assess biological variability. However, sample mixing after oligo-antibody labeling is only possible where high-quality antibodies can reasonably label all cells in a specific sample (*i.e.* pan-immune CD45 antigen[71]), and if the labeling technique does not lead to sample degradation[74]. For fragile or ultra-low input samples, pooling cells/nuclei from replicate animals in equal starting amounts before library generation can be a solution to sample biological variation, although replicate libraries should still be considered for statistical robustness.

Minimizing and/or accounting for batch effects.—Batch effects will occur when technical variation between sets of processed samples overshadows biological variation, in such a way that biological variation can no longer accurately be measured[75]. Batch effects are a notorious issue in 'omic' data, and often require additional computational pre-processing steps to correct[75, 76, 77]. To note, batch effects in 'omics' can occur at multiple steps, such as (i) original sample collection/storage, (ii) target enrichment (*e.g.* chromatin immunoprecipitation step, RNA isolation step, etc.), (iii) library construction step, or (iv) sequencing step (*e.g.* sequencing of replicate libraries on different lanes), and each batch layer can have compounded effects. A unique challenge in 'omics' studies with aging animals is that the timescale of experiments can span from days to years for longer-lived species (*e.g.* mice, humans). This feature can make it impractical to collect all biological samples in a very tight timeframe, which requires staggered study enrollment. Since correcting for batch effects may erase true biological signatures[78], whenever possible, aging 'omic' experiments should be designed to avoid batch effects. However, when batches are unavoidable (*e.g.* studies involving human samples or longitudinal collection), a proactive study design should enable downstream correction by ensuring that all (or most) biological groups are represented in each set of samples. In that case, computational algorithms can be used to correct 'omic' measurements before downstream analyses are performed both for bulk experiments (*e.g.* SVA[77], RUV[79]) or single-cell experiments (*e.g.* Harmony, LIGER[80]).

Accounting for global changes in aging 'omics'.—Algorithms for differential abundance analysis (*e.g.* DEseq2[81]) usually assume that most features are unchanged. However, when global changes in transcription, chromatin accessibility or levels of a chromatin mark occur, these assumptions are violated[82, 83, 84]. In this case, inclusion of exogenous molecules of the same type as those assayed can be included as "spike-in"

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controls (*i.e.* synthetic RNAs[82], chromatin from a different species[83, 84], etc.). Spike-ins can then be used to perform normalization before downstream analyses are carried out[79]. To note, in the absence of global changes in 'omic' landscapes, use of spike-ins will increase technical noise[85]. However, if they are unnecessary, spike-in molecules can be safely omitted from downstream analyses[4, 86]. Thus, if global changes are expected in response to aging, inclusion of spike-ins should be considered when possible.

Impact of tissue composition.—The cellular make-up of tissue can change with aging[87]. A common change stems from infiltration of immune cells in aged tissues[88, 89], which may drive general inflammation- and immune-related gene signatures upregulated with aging. Thus, if cell composition shifts are a concern in a tissue, it may be useful to pair bulk 'omic' analyses with analyses of cell type composition in this tissue at corresponding ages. Alternatively, transcriptional profiles of pure cell types that can be found in the cognate tissue, derived from atlas-type single-cell RNA-seq efforts or FACS-sorted cells (*e.g.* tabula muris, human cell atlas) may be used to perform transcriptomic deconvolution and detect shifts in cell composition (*e.g.* CIBERSORT, DeconRNAseq)[90]. Information on underlying cell composition may then be used as modelling covariates to prioritize the identification of cell-autonomous 'omic' changes in bulk tissue aging datasets.

Sample preparation methods.—For sequencing-based omics, selection strategies and library construction methods can broadly impact results. First, the selection strategies chosen for the assay should be carefully considered for each 'omic' layer: RNA species included in RNA-seq (*i.e.* polyA selection *vs.* ribo-depletion), specificity of the antibody used for ChIP-seq, sequencing of controls libraries (*e.g.* input or IgG immunoprecipitation), *etc.* In addition, for any sequencing-based methods, it is recommended to keep the number of PCR cycles as low as possible to limit the impact of bottlenecking or GC-biases. When this is difficult due to low input material, such as single cell RNA-seq, UMI-based profiling can help mitigate the impact of such biases. In the context of single-cell profiling, there are additional technical considerations related to sample preparation, compounded by considerations already outlined for bulk assays. Indeed, comparison of single-nucleus RNA sequencing to single-cell RNA-seq on human brain tissue revealed that genes related to microglia activation may be depleted in nuclei samples compared to whole cells[91]. Thus, it is crucial to use a uniform sample preparation method so that such preparation-related differences do not impact the results of comparisons across age groups. Importantly, since single-cell/nuclei RNA profiling can be very sensitive to ambient RNA in the preparation (derived from dead/dying cells or debris generated during cell/nuclei isolation), it may be important to estimate sample-wise ambient RNA levels and correct them (*i.e.* SoupX[92])., especially if aging could differentially impact cell viability. Finally, it is important to note that the process of cell dissociation for the purpose of single-cell profiling can artefactually induce stress signalling pathways. This issue is well documented in muscle tissue, where it is now recommended to fix the tissue prior to dissociation to single-cell profiling[93, 94], although it is likely relevant to other solid tissues requiring dissociation. Such technical issues are more likely to impact profiling of molecules with fast turnover (*e.g.* mRNA, phosphorylation events) than longer-lived molecules (*e.g.* proteins). However, the potential

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impact of dissociation should be taken into account when designing single-cell profiling "omics" experiments.

3. Analytical considerations for reproducible omics in aging and beyond

Due to variations in proposed algorithms to analyze various 'omic' experiments, as well as updates to algorithms, it is crucial to establish important rules for long-term reproducibility of 'omic' experiments. Although these are not exclusive to the aging field, some recommendations to promote reproducible 'omic' analyses are provided below.

Assessing data quality.—An important factor to enhance reproducibility in aging genomics experiments is to perform proper quality controls on the raw data to ensure data quality. The precise quality control metrics will depend on the underlying data. For example, for sequencing-based data it is important to ensure that the quality of raw reads is good (*e.g.* Phred > 20), that the average alignment rate to the reference genome is reasonable (>70%), and that alignment rate and sequencing depth are comparable across samples. For epigenomic datasets (e.g. ATAC-seq, ChIP-seq), additional quality control metrics should be considered (*e.g.* enrichment at transcription start sites, enrichment for specific binding motifs). Guidelines for quality assessment for quantitative proteomics[95, 96] and metabolomics[16] have been discussed elsewhere, and will apply to such datasets in the context of aging as well[97]. After raw data processing, dimensionality reduction techniques (*e.g.* PCA, MDS) can help visualize global similarities and relationships across samples, and can be powerful tools in detecting unwanted batch effects and (un-)expected patterns (*e.g.* separation as a function of age/sex).

Meta-data and covariate inclusion.—Because both biological and technical factors can influence 'omic' data collection, it is important to systematically include metadata in computational analyses, in addition to age groups (*e.g.* sex, genetic backgrounds, batch, library kit, RNA quality, etc.) (Table 2). This can help detect uncorrected batch effects and technical variation but should also be considered to enhance researcher's ability to detect true aging effects. If warranted, appropriate batch correction methods can be applied before proceeding to final data analysis. Evaluation of biological covariates (*e.g.* sex, genetic background, vendor, etc.) is also crucial at this stage. Indeed, non-linear interactions of such covariates with age can create analytical issues when using algorithms based on generalized linear models (*e.g.* DESeq2 Wald-Test[81]). In the case of non-linear interactions, alternative statistical tests should be used (*e.g.* DESeq2 Likelihood Ratio Test[81]).

Data filtering and result reporting.—Although specific pipelines to analyze aging 'omic' datasets should be selected to best fit specific experimental design, it is important to appropriately estimate false discovery rates [FDR] in analyses. For instance, a common practice has been to use log fold-change thresholding on differential gene expression analysis results after applying a significance threshold to reduce the number of significant genes to consider. However, when applied *a posteriori* to standard differential analysis methods (*e.g.* default DESeq2, edgeR), such post-hoc filtering leads to poor FDR control[98]. If gene expression change amplitude is crucial to analyses, tests specifically designed to determine differential expression with respect to a specific threshold should be used[98], or

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at least the null hypothesis should be amended to reflect this choice (as is possible in more recent versions of DESeq2). Alternatively, if researchers only wish to shorten the list of potentially interesting genes, test stringency can be increased by decreasing the FDR significance threshold (*e.g.* from 0.05 to 0.01). Finally, common pipelines should include filtering steps for regions known to yield technical artefacts in specific 'omic' types, *e.g.* ENCODE blacklist regions for epigenomic analysis[99] or mitochondrial genome for chromatin accessibility anaysis[100].

4. Reproducibility and validation in omics studies

Experimental validation of aging 'omics' studies.—There has been much debate, even beyond the aging field, about the need to "validate" the results of high-throughput omics studies (*e.g.* RNA-seq, ChIP-seq, proteomics) with low throughput methods (*e.g.* qPCR, Western Blot/ELISA). Historically, this practice stemmed from technical limitations of the first 'omics' method (*i.e.* cDNA microarrays): because microarray technology is based on hybridization of fluorescent cDNA to probes immobilized on a glass slide, followed by imaging, the technique suffers from poor sensitivity and accuracy in the low (*i.e.* below or equal to background fluorescence) and high expression (*i.e.* signal saturation) ranges[101]. Thus, the validation of top targets derived from 'omics' experiments on microarray platforms (*e.g.* cDNA, ChIP-on-chip) by qPCR (*e.g.* RT-qPCR, ChIP-qPCR) was widely adopted as a way to limit false positives that plagued the early days of microarray experiments.

In contrast, most of the technological limitations of microarray-based methods do not apply to sequencing-based methods, which demonstrate both exceptional sensitivity and specificity (*i.e.* robust detection of lowly and highly expressed genes)[102]. By force of habit, many believe that qPCR-based validation of sequencing experiments (*e.g.* RNA-seq) should be required for robustness. However, to be meaningful, external validation of an 'omics' dataset should only be done with a <u>superior</u> method (*i.e.* one with less technical shortcomings), or on <u>independent</u> samples/molecules. Unfortunately, as a specific example, RT-qPCR suffers from at least one major bias that makes it more noisy and less reliable than RNA-seq: reliance on normalization to "housekeeping" genes (*e.g. Actb*, *Gapdh*, *Hprt*, etc.), with the assumption that their expression is invariant across samples. However, such housekeeping genes can be very variable across conditions[103]. In addition, since only a handful of genes may feasibly be "validated" this way, sampling bias is such that it may not represent the reproducibility of the whole experiment. Thus, in general, RNA-seq or similar experiment types should not require qPCR validation[103, 104, 105]. A potential exception to this rule is if differences being are either very small or with large sample-to-sample variation in RNA-seq, and may thus be unduly influenced by unwanted technical noise. However, if qPCR is performed (*e.g.* to include independent samples in a different strain, or in a different ethnicity for human samples), the choice of normalizing amplicons should be informed by the 'omics' dataset itself to avoid introducing noise due to variable housekeeping gene expression. Similar considerations exist in the context for the validation of high-throughput quantitative proteomics by low-throughput Western Blot experiments, as standard housekeeping controls also vary at the protein level between conditions, leading to experts qualifying such validation as unnecessary and invalid[106, 107].

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Conceptually, one can also approach validation of omics studies on another level – are the predictions derived from the high-throughput dataset concordant with other measures? For instance, we believe that by cross-referencing biological measures across large datasets, multi-omic analyses are a good way to validate findings from one omics dataset (*e.g.* bulk *vs.* single cell RNA-seq, comparison with ATAC-seq, etc.). Although not necessary, the degree of validation would be increased if agreement across omics methods is derived from independent sample sets. Importantly, we recommend focusing on agreement at the level of enriched pathways/gene sets rather than specific genes, which will be more robust to exceptions. In addition, since omics is often hypothesis-generating, validation of functional predictions derived from the datasets in functional assays (*e.g.* differentiation potential of a stem cell, ability to clear protein aggregates, *etc.*) is also a good form of validation for an omics dataset. Finally, although not necessary, the validation of a few key genes at the protein level by western blot or ELISA would constitute another low-throughput option if validated/specific antibodies exist. However, we also note the usefulness of resource-type omics datasets, that can then be mined by the community for hypothesis generation (*e.g.* Tabula Muris Senis)[87]. In these cases, external validation may not be tractable at scale, but extensive quality-checking and meta-data recording should be done to make the datasets robust and reusable. Thus, different criteria of required validation should be considered if the dataset aims at hypothesis-generation *vs.* hypothesis-validation.

Reproducibility in aging omics research: sharing code and data.—Similar to ‘bench’ protocols, data processing and algorithmic choices are key to reproduce ‘omic’ results. However, since the format of methods sections cannot accommodate every coding detail, it is crucial for long-term reproducibility that all scripts used to analyze data always be deposited in free repositories (*e.g.* GitHub) or provided as supplemental zip files when corresponding manuscripts are evaluated. In addition, since different versions of the same tools or databases will provide different results, it is important that version numbers for all package/software and databases be provided for analytical reproducibility. Alternatively, the pipelines can also be packaged in ready-to-use formats, such as Conda environments or Docker images.

Most journals, like *Nature Aging*, now require deposition of the raw ‘omic’ data to public repositories (*e.g.* SRA, GEO, ProteomeXChange). Going further, we suggest that sharing intermediate processed data (*e.g.* count tables, peak files, etc.), although not mandatory, will help improve reproducibility and useability of ‘omics’ datasets (*e.g.* as part of a GitHub repository or as a supplemental archive). In contrast, for single-cell datasets, the final annotated object (*i.e.* including cell type assignments, meta-data, etc.) must be shared for long-term useability and reproducibility. This can be done readily by uploading relevant files on FigShare or Zenodo (*i.e.* text files of counts and metadata, RData object, etc.), or on dedicated single-cell browsing platforms (*e.g.* Broad Single cell portal; https://singlecell.broadinstitute.org/single_cell).

Conclusions

Our goal was to bring attention to best practices in designing and analyzing ‘omic’ studies in aging research. Many considerations that we highlight are generally applicable to ‘omic’

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studies in general, but we also discuss unique challenges related to aging research. In general, emerging evidence shows that many biological variables and technical choices can impact biological measures in sometimes unpredictable ways. Thus, we highlight specific sources of biological variation relevant to aging, which should be taken into account when designing aging 'omic' experiments (*e.g.* sex, reproductive status, genetic background, microbiome, circadian effects, etc.). Since aging experiments can span years, we also discuss the importance of accounting for technical variation and batch effects for aging 'omics'.

Some challenges in the use of 'omics' specifically in aging research remain open. For instance, since 'omics' experiments on older animals can show increased inter- and intra-sample variability [108, 109], addressing this inherent variability not as a mere confound, but potentially as a biologically relevant outcome, may provide important biological insights. Indeed, at least for single-cell modalities, algorithms are being developed to identify differentially variable genes[110]. Finally, 'omic' layers may become less correlated or decoupled with aging[44, 111, 112, 113, 114], creating unique challenges for multi-omic integration in the context of aging. In particular, decoupling between mRNA and protein levels has been observed broadly in post-mitotic tissues (*e.g.* brain [112, 113], heart [44], kidney[111]) or quiescent cells (*e.g.* muscle stem cells[114]). Proteins likely to accumulate in old tissues (*e.g.* extra-cellular matrix components, aggregation-prone proteins) may also be more susceptible to decorrelation than other proteins. Thus, robust and reproducible aging 'omics' should help the field better understand the systems biology of aging and age-related decline.

Acknowledgements

Some figure elements were created with BioRender.com. We thank Mr. Juan Bravo and Dr. Bryan Teefy for their insights on the manuscript. We apologize to those whose work was not cited due to space constraints.

P.P.S is supported by University of California, San Francisco and the UCSF Bakar Aging Research Institute. B.A.B. is supported by NIGMS R35 GM142395, NIA R01 AG076433, Simons Collaboration on Plasticity in the Aging Brain grant SF811217, Pew Biomedical Scholar Award #00034120, and the Kathleen Gilmore Biology of Aging research award.

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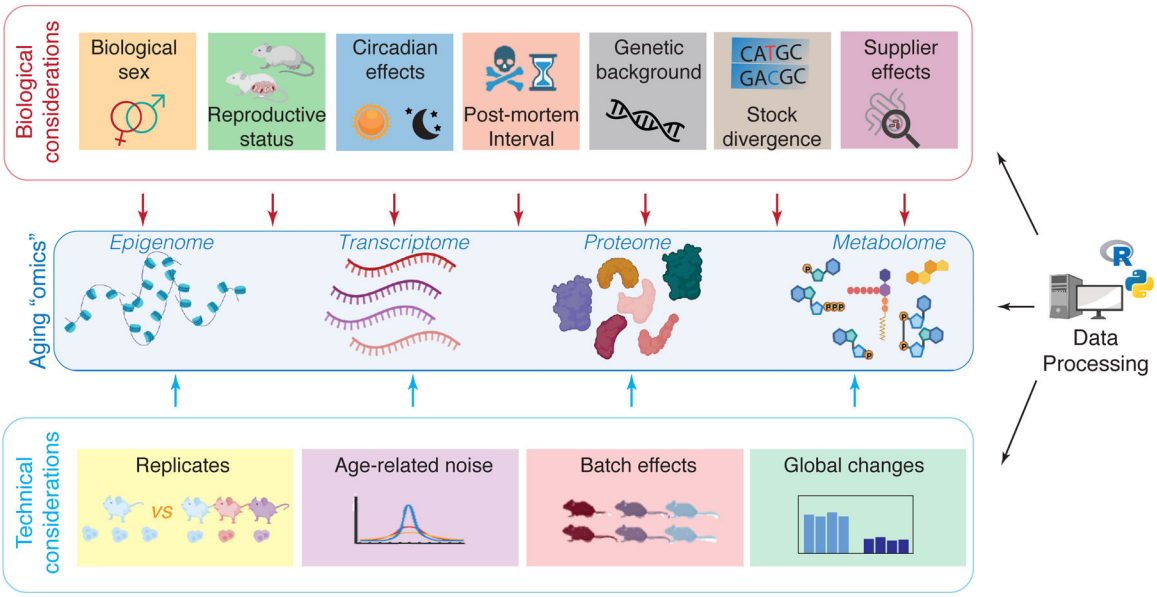

Figure 1: Considerations for the use of "omics" in aging research.

In addition to general considerations for "omics" experiments, aging "omics" requires additional care in experimental design and data processing due to unique challenges. Important biological variables can interact with aging signatures and should be controlled for carefully (e.g. sex). In addition, due to timescales involved in aging research, there are also important technical issues to take into account (e.g. batch effects). Both biological considerations and technical considerations should be taken into account during data processing to extract features of aging at the "omic" level. Some elements created with BioRender.com.

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Table 1:

Biological considerations in aging "omics"

Biological variable	Biological impact	Remedy	References
Biological sex	- X-linked and Y-linked gene expression - Sex hormone signaling	- Balanced representation of biological sex across groups	20, 21, 24
Reproductive status	- Sex hormone signaling - Immune remodeling	- Matching reproductive status across groups - Eliminate routine use of retired breeding	25, 26, 27
Circadian effects	- Circadian gene regulation	- Staggered collection approach	28, 29
Post-mortem interval	- Gene program activation post-mortem	- Staggered collection approach	30
Genetic background	- Changes in basal gene regulation - Impacts responses to longevity intervention	- Only compare animals with the same genetic backgrounds	34, 35, 51
Genetic divergence of inbred animal stocks	- Possible changes in gene regulation	- Only compare animals from the same genetic stock	37, 38
"Supplier" effects and microbiome	- Microbiome composition impacted by housing - Systemic changes linked to microbiome changes	- Only compare animals from the same supplier/animal facility	40, 41

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Table 2:

Metadata collection for aging "omics"

Variable	Sample-level recording	
	Required	**Optional**
Biological sex	- Biological sex	- Sex hormone levels
Reproductive status	- Mating status	- Estropause/Menopause - Estrus cycle phase (rodents)
Circadian effects	- Timeframe of euthanasia	- Day/light cycle of animal facility
Feeding and metabolism	- Chow - Weight	- Fasting period
Genetics	- Strain and supplier	
Pathology	- Gross pathological findings	- Pictures at euthanasia
Batch processing	- Sample set processing	- Euthanasia date - Processing date
Collection scheme	- Synchronous or asynchronous for age groups	- Relatedness (if non inbred)
Replicates	- Number of animals per sample - Labeling of technical replicates (if any)	- Additional per animal information if animals were pooled
Processing pipeline	- Software versions - Genome build - Code and processing scripts	- Intermediate processing files