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Stress relief: emerging methods to mitigate dissociation-induced artefacts

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Glossary

-dissociation-induced artefacts: modifications of the molecular profile of a cell induced during its enzymatic and mechanical dissociation. Most often, these refer to transcriptional changes since they are the most extensively studied, yet histone modifications, like H3K27ac, and post-translational protein modifications have also been reported.

-single-nucleus RNA-seq: RNA sequencing method for profiling gene expression in extracted nuclei. This method relies on direct extraction of nuclei from tissue and does not involve a cell dissociation step. In addition, it allows to recover and analyse nuclei from syncytia, like the myofibres, which are not accessible by standard cell isolation techniques.

-early-response genes: a group of genes that are rapidly induced, usually within minutes, in response to a variety of environmental stressors. The earliest known and best characterized include *fos*, *myc* and *jun*, amongst others.

-psychrophilic proteases: also known as *cryophilic*, are proteases that demonstrate enzymatic activity at temperatures as low as 6°C. These cold-active proteases derive from organisms thriving at near-zero temperatures, like the soil bacterium *Bacillus licheniformis* from Himalayan glaciers.

-Thiouracil (TU)-tagging: a method for covalently labelling newly transcribed RNA in vivo. Tagging is achieved by the uracil phospho-ribosyltransferase (UPRT) enzyme together with 4-thiouracil (4TU), which acts as a substrate. The thio-RNA is biotinylated and streptavidin beads can be used for purification from complex tissues. Transgenic animal models expressing inducible UPRT have been developed, which allow isolation of de novo transcribed RNA from specific cells.

-in situ Fixation (iSiFi): a method to capture the molecular profile of cells as they exist in their native tissue context. This method is based on paraformaldehyde fixation of cells in the tissue prior to the mechanical/enzymatic dissociation. The recovered cells have been shown to be compatible with RNA-seq, ChIP-seq and reduced representation bisulfite sequencing (RRBS) for DNA methylation.

Abstract

The rapid progress of single-cell RNA-sequencing at large scales has led to what seemed impossible until recently, the generation of comprehensive transcriptional maps of nearly all cells in multicellular tissues. We pinpoint three key elements as critical to the production of these maps: scalability, spatial information, and accuracy of the transcriptome of the individual cells. Here, we discuss the ramifications of traditional cell-isolation protocols when capturing the transcriptional signature of cells as they exist in their native tissue context, the methods that have been developed to avoid these distortions, and the biological processes that have unravelled on account of these upgraded methodological approaches.

Impact of isolation techniques on the molecular profile of a cell

Since the first description of a cell [1] and the formulation of its theory [2], many cellular attributes have been employed to define cell types in multicellular organisms such as shape, anatomical location and function. With the emergence of biology's central dogma [3], the macro-molecular content of a cell, such as its RNA or protein composition, became preponderantly used for cellular ontology segregation. Among them, the transcriptomic profile of a cell has received the most attention from modern cellular biologists. Despite the variable RNA to protein correlations [4], the transcriptome of a cell is a very convenient proxy of its proteome and has been studied extensively, notably via single-cell RNA-sequencing (scRNA-seq), to infer lineage identity, active biochemical pathways and cellular functions. Such inferences have allowed researchers to match physiological and pathological states with the activity of specific genes and pathways as well as segregating cells in remarkably elaborate subgroups. The amount of high-throughput transcriptional maps published is expanding at a restless pace and recently, large scale projects, such as the *Human Cell Atlas* or murine cellular atlases, undertook the daunting task of systematically sequencing all cells from mammalian organisms [5-7].

Every cell in a multicellular organism is in constant interaction with its local microenvironment that contains both cellular and non-cellular components, including secreted growth factors, the extracellular matrix, circulating hormones, nutrients and oxygen. Cell isolation protocols drastically disrupt the cellular microenvironment and are based on a combination of mechanical dissociation and lengthy enzymatic treatments, often at 37°C. In light of these facts, the hypothesis that the mere process of isolating a cell from its niche significantly alters its gene expression profile appears reasonable.

One of the first reports on how sample preparation could alter transcriptomic data came from an early microarray-based study, reporting that human peripheral blood cells stored at room temperature overnight induced the expression of thousands of genes, including early-response genes [8]. A decade later, reproducibility difficulties between laboratories in fluorescence-activated cell sorting (FACS) profiles and efficiency of organoid formation from human breast cell preparations were reported [9]. After carefully comparing protocols, the authors found that length and agitation frequency of the proteolytic enzyme collagenase during the digestion step explained the experimental discrepancies. This finding was confirmed at the transcriptional level by the induction of stress-related genes in dissociated neurons [10] and later in muscle stem cells (MuSCs) [11–13] and kidney cells [14]. Since then, virtually all cell-types have been shown to express a characteristic stress signature given sufficient dissociation time.

Emerging methods for capturing transcriptomes as they exist in their native tissue context.

Several groups across the world undertook in parallel the task of circumventing **dissociation-induced artefacts** in high-throughput studies. These efforts resulted in a handful of emerging methods, each with their own strengths and weaknesses. Such methods can help elucidate the impact of dissociation and the prevalence of cellular stress across a broad range of biological conditions, organisms and cell types (Table 1).

Direct extraction of nuclei from tissues for single-nucleus RNA-seq

It goes without saying that an obvious strategy to avoid the impact of dissociation is to exclude it from the experimental protocol. In that spirit, single-nucleus RNA-seq (**snRNA-seq**), which relies on direct lysis of intact tissues for extraction and barcoding of single nuclei [15–17], proposes several advantages over other methods for large-scale projects [18].

A seminal study by B. Lacar and colleagues demonstrated that hippocampal neurons isolated by standard procedures were highly expressive in **early response genes** *Jun*, *Arc* and *Fos* [10], a puzzling result considering that products of these genes were almost undetectable by immunofluorescence on resting hippocampal neurons *in vivo*. This prompted the authors to use snRNA-seq and demonstrate that the presence of the early response genes was in fact an artefact of the dissociation procedure and that unstimulated neurons do not express them. This discovery was confirmed by snRNA-seq in the mouse visual cortex [19], kidney [20, 21], and in the liver and skeletal muscle [22].

Of importance, it has been shown that the nuclear transcriptome largely reflects its whole-cell counterpart [18]. However, some discrepancies are observed, including the identification of more intronic sequences from unsliced pre-mRNA, which might be reflective of the *de novo* transcript production, and the failure to capture cytosolic RNAs, including miRNAs and lncRNAs. Moreover, in the context of skeletal muscle and liver, snRNA-seq detected the expected cellular populations, albeit with different relative abundances compared to RNA-seq on dissociated cells. Specifically, we found an under-representation of endothelial cells in snRNA-seq datasets from intact tissues: endothelial/MuSCs ratio was 6.2 for nuclei derived from the dissociated cells, and 0.7 for nuclei extracted from intact tissue [22]. This bias is likely due to differences in extraction efficiency of cells from intact and dissociated tissues. Careful histological studies on biopsies combined with flow cytometry methods will help determine the actual population ratios. Multinucleated muscle fibres are another example of how cell morphology can impact the representation of tissue composition. Myofibers are large, elongated multinucleated syncytial cells and by far the main cell type of skeletal muscle (roughly 55% of all the nuclei of skeletal muscles, based on snRNA-seq) yet are absent in single-cell RNA-seq (scRNA-seq) atlases, as it is not possible to isolate and sequence them using standard protocols. Recently, implementation of snRNA-seq on skeletal muscles has uncovered myonuclear sub-populations and coordinated transcriptional properties of the myonuclei that previously were not accessible for analysis [23–25].

Altogether, these results indicate that snRNA-seq is an advantageous method in preventing dissociation-induced artefacts and providing access to the nuclei of syncytia, whilst still enabling large-scale transcriptional profiling experiments with marginal costs and complexity. Nevertheless, the impossibility to select for cell-surface antigens, the lack of cytoplasmic RNAs and a lower sensitivity that could impede the detection of low expressed genes add some limitations to this method.

In situ cell fixation prior to dissociation

Aldehydes have been used for more than a century to preserve biological samples through the process of fixation [26]. Fixation preserves organic samples and arrests any biological function, including transcription, by cross-linking the macromolecules [27]. Paraformaldehyde (PFA) has been shown to preserve RNA integrity and composition, notably for RNA-seq [11], [28]. Independent studies employed PFA fixation to preserve the nascent transcriptome of isolated cells and abrogate the impact of the dissociation procedure [11, 12]. Cells were fixed within the tissue of interest prior to dissociation with a low-concentration of 0.5% PFA, in a process that we termed *in Situ Fixation*, or *iSiFi* for short [11]. Using bulk RNA-seq on *iSiFi*-treated skeletal MuSCs we made the unexpected observation that close to half of the transcriptome was dysregulated during isolation, with thousands of both upregulated and downregulated transcripts. Importantly, *iSiFi* efficiently preserved cells' transcriptome and prevented the induction of early-response genes. These results were later confirmed at the protein level and were expanded to describe specific biological functions, as discussed later [22], [29, 30]. Recently, the fixative glyoxal has shown some superiority in term of RNA-quality for transcriptional studies after cell sorting [31]. Single-cell studies, methanol has also been used as a fixative agent to preserve the transcriptome of mouse kidney cells [21] and mouse inner ear cells, successfully allowing the identification of rare cell types, notably spindle and root cells [32]

The use of fixation prior to dissociation for gene expression preservation is a low-cost procedure, using accessible chemicals with minor modifications to existing protocols. *iSiFi*-treated cells are further compatible with chromatin immunoprecipitation (ChIP) for analysis of histone H3 modifications and specific transcription factors, and DNA methylation analysis by reduced representation bisulfite sequencing (RRBS) [11], [33]. Of note, however, to date PFA-fixed cells are not compatible with high throughput single-cell platforms, such 10XGenomics, while methanol-fixed seems to be for some cell systems [34].

Cell dissociation with cold active proteases

An alternate approach to mitigate dissociation-induced modifications is by performing tissue dissociation at low temperatures using cold-active enzymes to decrease cellular activity and arrest transcription. An elegant study by Adam et al. confirmed this notion by using a **psychrophilic protease** isolated from the Himalayan glacier bacterium *Bacillus licheniformis*, to digest developing mouse kidney tissue for 15 min at 6 °C with the serine protease subtilisin A [14]. Since then, several studies reported similar findings using cryophilic proteases. In mouse ovarian and breast tumours, a core set of 512 genes induced by standard procedures was identified using a similar cold digestion protocol [35]. Furthermore, in adult murine hippocampus, low temperature mechanical dissociation at 4°C was also shown to prevent the induction of stress response genes [36]. Comparable findings were attained in the murine kidney [21], retina [37] and inner-ear [38].

To date, our understanding of the cold-shock response in eukaryotes is limited. Several studies have demonstrated that exposing mammalian cells to sub-physiological temperatures invokes a coordinated cellular response involving induction of cold-shock proteins and modulation of translation, metabolism, cell cycle and the cell cytoskeleton [39]. Therefore, although cold digestion prevents, to a large extent, the dissociation-induced artefacts, it is still possible to alter in some way the *in vivo* molecular profile of the cells, for example, cryopreserved cells experiment some induction of early-response genes [40]. Of note, cells isolated using standard procedures also commonly experience cold-stress during several steps of the protocol, such as centrifugation and transfer of cells, as these steps are performed at 4°C and on ice, respectively. Cold digestion is one of the few procedures described here that keeps the cells alive, which can be a critical asset for downstream applications.

Furthermore, cold digestion is compatible with standard RNA extraction and sequencing kits including 10xGenomics, without protocol specific adaptations. The use of cold active proteases represents a promising avenue toward stress-free transcriptional studies and determination of the range of tissues in which it could be effectively applied to, would be of great interest.

Inhibitors during the cell dissociation

Similar to cold digestion, slowing down or blocking the transcriptional machinery with inhibitory compounds has the potential to reduce experimentally introduced changes. Digestion of mouse skeletal muscle in the presence of α -amanitin, a selective inhibitor of RNA polymerase II and III, was successfully associated with a lower dissociation-induced signature [12] and in the mouse brain, the potent transcriptional inhibitor actinomycin D elicited similar results [38, 39]. In another study, a cocktail of chemicals aimed to block calcium entry, neuronal activity, transcription and translation also prevented dissociation-induced genes expression [43]. However, these methods target modifications driven by neo-transcription, but miss other alterations such as gene repression, post-transcriptional modifications and more. Furthermore, it is difficult to ensure total and homogeneous diffusion of the chemical inhibitors in tissues, as well as their specificity. In this regard, we recognize the potential to mitigate the dissociation-induced signature by these methods, but we believe that more studies are necessary to assess the activity of alternative RNA regulation mechanisms under transcriptional inhibition mechanisms, and the extent to which the generated atlases will be free from artefacts.

Cell-type specific nascent RNA tagging

Conditional labelling of nascent RNA in specific cell types is a sophisticated tool to study gene expression *in vivo* [41, 42]. Such methods can be applied to avoid dissociation-induced artefacts when the induction of RNA tagging precedes the cell-isolation procedure. For example, **Thiouracil (TU)-tagging** induced with the MuSC-specific *Pax7-CreERT2* recombinase driver, permitted the isolation and sequencing of the quiescent transcriptome, free from artefacts [12]. Ribosomal-tagging was similarly used in mouse microglia [46] and inner-ear [38], [47] to label RNA prior to cell dissociation and thus dampen the effect of the procedure. These methods are precise and elegant, allowing the spatial and temporal control of RNA labelling *in vivo*. However, due to the complexity of these procedures they are difficult to implement in diverse biological systems.

Post hoc bioinformatics correction of dissociation-induced modifications

Considering the intellectual and financial investments that are injected into ongoing and published transcriptomic studies, it would be widely useful if the dissociation-induced modifications could be retrospectively attenuated or completely removed from existing datasets. Indeed, it is tempting to postulate that given a sufficient data volume, reducing these alterations computationally would be possible. Most recent studies have relied on complex machine learning algorithms, both unsupervised and supervised mutual nearest neighbours detection, to untangle and correct biological variation from technical noise [45, 46]. These tools perform under the assumption that biological variability (e.g., the difference between cell types) is much greater than batch effect-induced variability (e.g., technical noise due to the preparation method).

Several groups took the challenge of correcting for dissociation-induced artefacts *in silico*. Some studies removed cells expressing high stress signatures from downstream analyses; this accounted for approximately 10% of the cells in a study done on human prostate cells [50]. Other studies removed only stress genes [48–53], both stress genes and stressed cells [57], or regressed out dissociation-induced genes as a confounding variable from their analyses [55, 56]. Most of the studies cited above

cleaned their datasets by excluding a limited set of less than 200 dissociation-induced genes, based on an earlier study on MuSCs [13], whilst the literature suggests that 10-50% of the transcriptome becomes differentially expressed by dissociation [22]. Ironically, the proportion of a cell's transcriptome altered by dissociation is so large that trying to regress the stress artefact out of it might alter it even further, creating artificial reference maps, distant from the *in vivo* profile.

Recent systematic and temporal analysis on dissociation-induced stress [22] suggests that given enough time every cell gets altered by the dissociation procedures, and that each cell-type reacts idiosyncratically and dynamically to it. Moreover, most batch effect corrections assume orthogonality in the biological sub-space, i.e., that all cells within the same experiment undergo the same batch effect. For these reasons, it appears technically challenging to infer for each cell-type, in each biological setup, the optimal gene adjustment required to remove the specific stress stimuli along a dynamic stimulation trajectory.

In our opinion, corrections *a posteriori* for noise factor should only be considered as a last resort since specialized tools now exist, presented in this review, to prevent it. Nevertheless, *in silico* corrections can be a helpful tool to improve existing datasets if a cell-type specific, biologically adapted set of tools is available for correction.

Spatially resolved transcriptomics

Despite not having systematically studied dissociation-induced genes, it is explicit that spatial transcriptomics methods performed on tissue sections [57–60] will preserve the cells from dissociation-induced modifications. These methods, comprehensively reviewed elsewhere [64], were designed to perform high-throughput transcriptomic profiling of cells *in situ*, therefore, conserving precious topological information about tissue architecture, cell interactions and transcript localization without requiring cellular isolation. To a lesser extent, laser-capture microdissection on frozen samples coupled with next generation sequencing is an adapted approach for retrieving transcriptome information linked to an anatomical location, whilst circumventing dissociation-induced artefacts. Examples exist in human and mouse brain microvessels [65], as well as on single neurons by aspirating the cytoplasmic content of a neuron following electrophysiological measurements [66]. To date, these methods do not exhibit the simplicity, scalability, resolution (often not single-cell), sequencing depth, genome coverage and ecosystem of analysis tools necessary to substitute cell-isolation based methods. Unquestionably, however, spatial transcriptomics will become the technology of choice for transcriptomic studies, as the breadth of knowledge stemming from the topological information is unmatched.

Collectively, most of the methods described in this review are easily implemented. Nevertheless, a simple guideline for stress-free preparations is described in Box 1.

Case studies: how dissociation-induced artefacts can mask dynamic biological functions

Comparative studies might be mostly spared interference by dissociation, assuming that the artefacts are equivalent between conditions and hence cancelled out (Figure 1A). In studies aiming to represent the absolute profile of cells, such as in reference atlases, dissociation-induced modifications are readily detected, however, they retain adequate accuracy to yield comprehensive results (Figure 1B). The strongest interference due to dissociation-induced artefacts, in our view, has to do with the interpretation of biological functions in dynamic cellular systems. Many processes take place in a timeframe that is an order of magnitude shorter than tissue dissociation itself, such as signal transduction, transcription factor binding or membrane depolarization. Here, in Box 2 and Box 3, two case studies are described that demonstrate how the methods presented above can be harnessed to

circumvent dissociation-induced stress and give insight to previously inaccessible biological processes (Figure 1C).

Concluding Remarks and Future Perspectives

We presented here currently available methods to circumvent dissociation-induced artefacts including cold-dissociation, snRNA-seq, *in situ* fixation, the use of chemical inhibitors, nascent transcriptome pull-down, bioinformatic *post-hoc* corrections and spatial transcriptomics. This review aims to provide a roadmap for stress management in high-throughput transcriptional studies. Far from perfect, every experimental intervention brings its own set of trade-offs, artefacts, and complications (see Outstanding Questions). Therefore, a careful analysis of the benefits and risks of each method is necessary for choosing for each project the most adapted procedure, which will yield the most accurate transcriptional maps.

This review serves as a cautionary tale of data interpretation on cells isolated from their *in vivo* environment. More importantly, it aims to promote the development of more sophisticated methods to accurately capture single-cell states as they exist in their native tissue context and towards the construction of second-generation transcriptional atlases.

Box 1. A simple guideline for stress-free preparations.

The future gold-standard for transcriptomic studies will be free of dissociation, in the form of spatial transcriptomics methods. However, to this day, such methods remain out-of-reach for most projects. A simple, practical guideline stemming from our work is to quickly dissociate the tissues, whenever possible. Time-course experiments in muscle stem cells and systematic analysis of published literature in diverse tissues, indicate that digestion time of 30 min or less induced only marginal transcriptional alterations [22]. Of note, notwithstanding cell type differences, the time required for FACS seems to be less important as the cell isolation procedure has been reported to have only a minor impact on the transcriptome [67]. The majority of tissues from the Tabula Muris cell atlas [5] escape encounters of dissociation-induced artefacts through this simple route, demonstrating how well-managed, methodological practices can yield great results. When celerity is unattainable, however, efforts must be directed towards preserving cells' *in vivo* molecular state using the methods described in the present review. Regardless of the experimental strategy, downstream datasets must be systematically evaluated for signs of transcriptional stress, and bioinformatic interventions could be employed to reduce data distortions.

Box 2. Cell dissociation fires neuronal signals that overlap with activity-dependent transcription.

A prominent example of interference of data acquisition and experimental methodology comes from neurological studies on activity-dependent transcription. Discovered almost 40 years ago [68], *Fos* became the prototypical activity-regulated gene. Like the rest of its gene family, *Fos* becomes activated by calcium signalling, is a reporter of neuronal activity (e.g., membrane depolarization), plays a role in engram formation, synaptic plasticity and long-term adaptation, and is also a dissociation-induced gene [69]. How can one correlate the transcriptional response to each one of these processes given their respective kinetics -from minutes to days- and the distorting impact of dissociation? When B. Lacar and colleagues used standard single-cell RNAseq to evaluate the transcriptome of dissociated hippocampal neurons exposed to an epilepsy-inducing drug (PTZ), they scored, as expected, high levels of *Fos*. However, they observed that *Fos* and other activity-dependent genes were also detected in their untreated control group, in direct contrast to *in situ* hybridization experiments showing virtually no such transcripts in resting hippocampal neurons [10]. Only when the team employed single-nuclei RNAseq were they able to circumvent this caveat and uncover the actual activity-dependent transcriptomic signature in the hippocampal neurons in response to a novel environment. Harnessing this method allowed researchers to generate an accurate snRNA-seq transcriptomic atlas of more than 18,000 neurons, which delineated a continuum of heterogeneous activation states [70]. In a separate study, the rapid transcriptional response of neurons to light stimulation was investigated [43]. However, researchers found that the dissociated neurons from mice that were kept in the dark exhibited a strong activity-dependent profile similar to that of the light-stimulated group, preventing any meaningful interpretation. When the researchers applied a cocktail of chemical inhibitors to dampen cellular activation, a light-stimulated transcriptional program was uncovered together with cellular heterogeneity in response to this stimulation, both between and inside cortical layers [43], further emphasizing the importance of mitigating of dissociation-induced artefacts for the precise study of the biology of neurons.

Box 3. How skeletal muscle stem cells quiescence was escaping us for years.

A paradigm of how specialised methods can help us uncover up-to-date inaccessible biological processes and introduce new concepts, stems from studies on quiescence exit of skeletal MuSCs. These cells, like all quiescent stem cells, have the capacity to stay in a reversible G0-arrested state, whilst retaining the ability to regenerate muscle tissue upon injury [71]. In accordance with the work on neurons presented above, initial transcriptomic studies on freshly isolated MuSCs from resting muscle found strong enrichment of early-response genes and pro-mitotic oncogenes. This conundrum puzzled the field for years and it was hypothesized that skeletal MuSCs were molecularly poised for activation in response to injury [72]. By *iSiFi* and snRNA-seq studies, it was later established that freshly-isolated MuSCs were in fact prematurely activated upon isolation and transcriptionally estranged from quiescence [11, 12], [22]. Moreover, these and subsequent studies enabled the unveiling of biologically relevant determinants of MuSC activation, including the rapid upregulation of genes encoding glycolytic enzymes at the expense of fatty-acid oxidation transporters and enzymes [11], [73], the global increase of transcript splicing via the phosphorylation of DEK [30], and the induction of polyamine biosynthesis pathway via the induction of ornithine decarboxylase (*Odc*) [22]. These studies defined a new “early activation” state of MuSCs [11, 12], [22], [29, 30] and found that during this step, an essential ERK1/2 primary proliferation signal preceded the initiation of the Notch-regulated myogenic program [22]. By combining ERK1/2 MAPK inhibition with Notch signalling induction, *ex vivo* skeletal MuSCs were maintained *ex vivo* in a proto-quiescent state [22], underlining the functional relevance of the improved transcriptome methods presented here. Finally, these results were confirmed through the genetic impairment of dissociation-induced responses in MuSCs by knocking-off the early-response gene *Fos*, which impaired their regenerative capacity [74].

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Declaration of Interests

The authors declare no competing interests.

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Table 1. Summary of available methods for mitigating cell dissociation-induced modifications of the transcriptome.

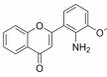
	 Cold digestion	 Fixation	 Single-nucleus RNA-seq	 Inhibitors	 RNA tagging	 Spatial transcriptomics	 Bioinformatics
Accuracy	High	High	High	Medium	High	High	Medium
Affordability	High	High	High	Medium	High	High	High
Accessibility	High	High	High	Medium	High	High	High
Cell viability	High	Not applicable	Not applicable	High	High	Not applicable	Not applicable
RNA yield	High	Medium	Medium	High	High	High	Not applicable
Cell number	High	High	High	High	Medium	High	High
Strength	Preserves cells Reversible	Low-cost and simple	Low-cost and simple	Preserves cells	Nascent transcription s-/t- control	RNA topography	Post-hoc correction
Weakness	Potential impact on transcriptome	Not compatible with 10x (PFA)	Nuclear transcriptome	Penetrance Specificity	Transgenics limitations	Equipment Coverage	Data distortion

Figure 1. Impact of cell dissociation-induced modifications on single-cell studies.

