Ideally, cells destined for RNA-seq analysis should be fixed before removal from growth media or tissue context as tissue dissociation and cell staining methods induce st3

RNA from formaldehyde-fixed material [17], however covalent crosslinks must still be reversed by heat treatment to remove adducts that otherwise inhibit enzymatic reactions: peptides remain attached after proteinase K digestion, and even once these are removed polymerases can still be inhibited by residual methylol groups, accounting for the poor amplification of DNA and RNA from formaldehyde-fixed material [16, 18, 19]. Furthermore, these residual adducts inhibit base-pairing and so will certainly impede purification of poly(A)+ RNA by binding to oligo(dT), a key step in many bulk and single-cell mRNA-seq protocols [20, 21]. Although crosslinks and residual adducts can be removed by heating, balancing sufficient crosslink reversal against thermal degradation of RNA is challenging [16, 22, 23].

Of course, other fixatives are known that do not crosslink RNA to protein or introduce stable adducts. We and others routinely employ ethanol fixation on yeast prior to RNA isolation and find the fixation speed and preservation of RNA to be excellent [24, 25], while similarly high quality RNA has been isolated from methanol or ethanol-fixed mammalian cells [26±29]. Another option is glyoxal, a protein crosslinking fixative that performs similarly to formaldehyde in most applications [30±32]. Glyoxal, like formaldehyde, has long been used as an RNA denaturant in gel electrophoresis so it is known to maintain RNA integrity [33], and has useful characteristics including reduced toxicity and increased stability in solution. Importantly, although glyoxal forms protein-protein crosslinks with a similar efficiency to formaldehyde, glyoxal reacts very differently with nucleic acids; only guanine reacts to any measurable extent with glyoxal and in doing so creates a stable heterocycle that does not form covalent crosslinks with proteins under normal conditions [34] (Fig 1B). Furthermore, the guanine-glyoxal heterocycle is unstable at pH>7 and so inhibitory adducts rapidly dissociate under standard buffer conditions [34].

Non-RNA-protein crosslinking fixatives therefore have the potential to be extremely useful in transcriptomic studies of flow sorted cells. However, little is known about the survival of RNA in alcohol fixed cells during downstream staining and sorting procedures, and to our knowledge the survival of RNA in glyoxal fixed cells has not been investigated. Here we show that glyoxal fixation allows extraction of high-quality RNA using standard protocols from stained and sorted cell samples, and that this RNA can be processed into high quality RNA-seq libraries.

Results

Optimisation of staining steps for RNA extraction

To determine the effectiveness of ethanol fixation in preserving intact and accessible RNA in mammalian cells, we extracted RNA from COLO205 cells that were either unfixed, fixed with 70% ethanol or fixed with 4% formaldehyde. RNA extraction was performed using TRI reagent, a monophasic GTC-phenol RNA extraction solution, and 20% of the RNA obtained was analysed on denaturing RNA mini-gels. Plentiful high quality RNA was obtained from the unfixed cells, whereas we did not recover detectable RNA from formaldehyde-fixed cells which was as expected given that proteinase K digestion was not performed (Fig 2A, compare lanes 1 and 3). A high yield of total RNA was also obtained from ethanol fixed cells but this RNA was partially degraded (Fig 2A, compare lanes 1 and 2), and similar problems were observed with 100% methanol fixation (Fig 2B, lanes 1±3). We suspect that degradation occurs because alcohols permeabilise membranes and allow extracellular and intra-organellar ribonucleases to enter the cytoplasm and degrade RNA before these enzymes are denatured. Whatever the reason, this partial degradation was problematic as the extent of degradation varied between experiments, and is also likely to be variable between cell types and tissue samples depending on local and intracellular RNase concentrations.

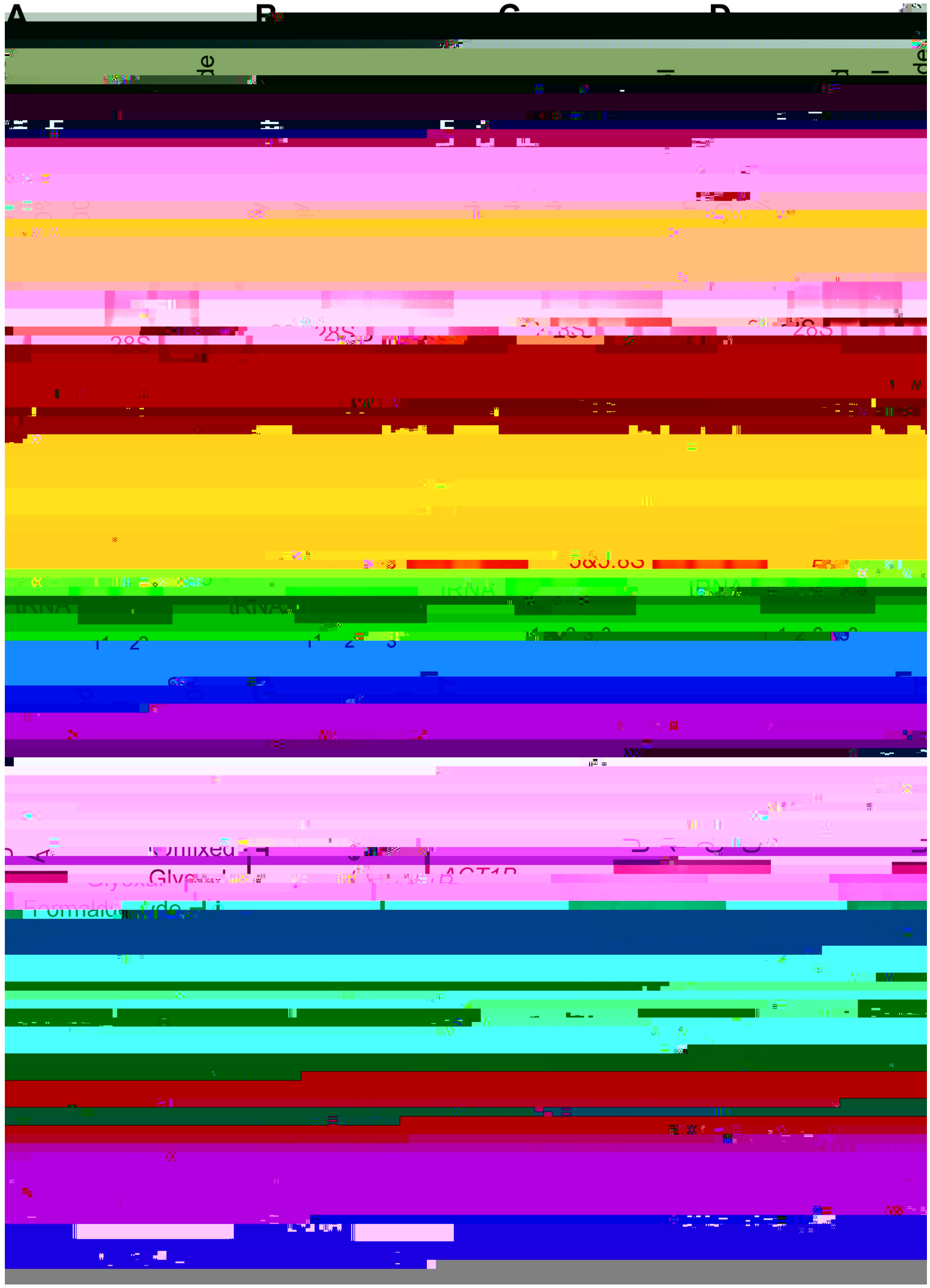

Fig 2. Determination of RNA-compatible fixation and permeabilisation conditions. A: 1x10^6 COLO205 cells were either unfixed (lane 1), fixed with 70% ethanol on ice for 15 minutes (lane 2) or fixed with 4% formaldehyde on ice for 15 minutes (lane 3). Unfixed cells were dissolved immediately in TRI Reagent, fixed cells were washed once in PBS by centrifugation at 2000 x *g* for 3 minutes at 4ÊC before RNA extraction with TRI Reagent. 20% of RNA obtained was separated on a 1.2% glyoxal gel and imaged by ethidium bromide stai[illegible] 1.9133 0 Td (RNre)Tj 2.1118 0 Td (dither)Tj -42.58 0 T

Since alcohol fixation did not preserve RNA well in our cell-line of choice, we turned to glyoxal fixation. Two formulations of acidic 3% glyoxal fixative have recently been validated for immunostaining, either with or without 20% ethanol [30], and TRI Reagent extraction yielded RNA of excellent quality from COLO205 cells fixed with either glyoxal formulation (Fig 2C). All further experiments were performed with glyoxal fixati2C

staining for intracellular antigens may not be compatible with recovery of small RNAs and miRNAs.

To confirm that high RNA quality and yield are maintained after staining, we then compared RNA from cells lysed directly in TRI Reagent after harvest against RNA from cells subjected to glyoxal fixation and methanol permeabilisation followed by a 2-step primary and secondary antibody staining procedure. The quality of RNA was equivalent between the stained and unprocessed cells and yield only slightly reduced (Fig 2G). We therefore find that glyoxal fixation followed by methanol permeabilisation allows immunostaining of intracellular antigens and recovery of high quality RNA by standard methods without requirement for proteinase digestion or de-crosslinking.

Glyoxal fixed cells yield high quality RNA-seq libraries after flow sorting

We chose Cyclin B1 (CCNB1) as a trial intracellular antigen for validation of RNA-seq analysis in glyoxal fixed and stained cells. CCNB1 accumulates during G2 phase of the cell cycle and is degraded at the end of M-phase, so the quality of staining and sorting based on CCNB1 can be verified by comparison to DAPI staining for DNA content. Furthermore, gene expression changes across the cell cycle are well characterised and so the successful purification of G2/M-phase cells should be detectable based on differential expression of cell cycle genes in RNA-seq data.

To determine whether our staining protocol reproducibly maintained RNA quality, we compared cells immediately dissolved in TRI Reagent at harvest to cells that underwent staining. Antibody staining was performed according to the consensus protocol given in the Materials and Methods section entailing glyoxal fixation, methanol permeabilisation and two-step immunostaining with rabbit anti-CCNB1 followed by Alexa Fluor 488-labelled donkey anti-rabbit. Two replicate experiments were performed for COLO205 cells and two for MCF-7 cells, RNA was extracted with TRI reagent and RNA quality assessed by Bioanalyzer. Representative Bioanalyzer plots are shown (Fig 3A) and details of RNA integrity and yield are given in Table 1. The RNA integrity number (RIN), which varies from 1±10 was passable for RNA from stained COLO205 cells (>7.5) and very high for RNA from stained MCF7 cells (9.5). Importantly, there was little reduction in RNA quality during staining (the worst outcome was a reduction in RIN of 0.9 in one COLO205 replicate). Furthermore, extractions were efficient with >40% of the RNA yield obtained from stained cells compared to directly harvested cells.

We then generated RNA-seq libraries from each of these RNA samples using a NEBNextthe

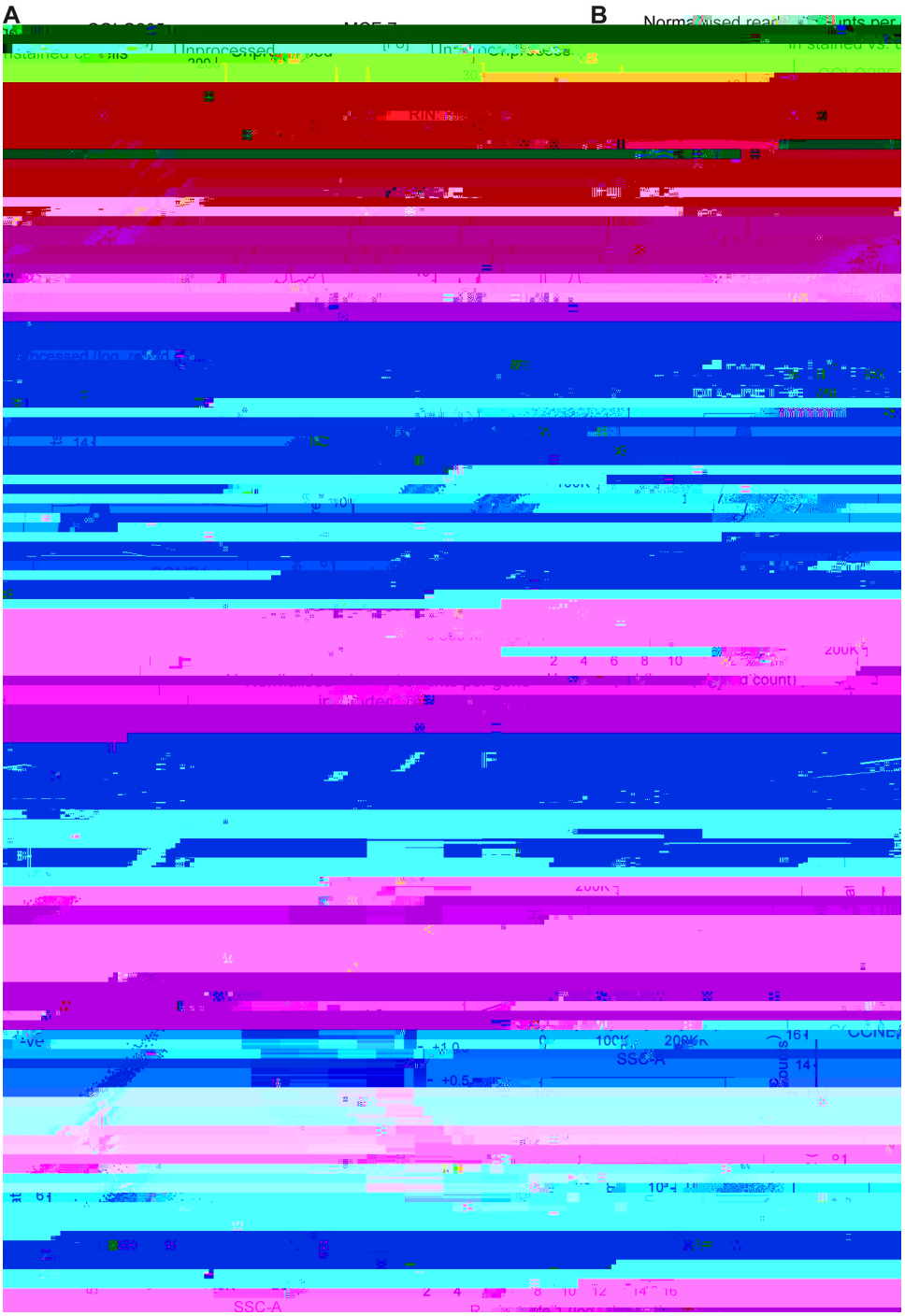

Fig 3. RNA-seq of stained and sorted cell populations. A: Bioanalyzer profiles of total RNA derived from unprocessed cells (harvested directly from trypsinised cells) compared to cells that have undergone glyoxal fixation, permeabilisation with 100% methanol and indirect immunofluorescence for CCNB1. 0.1±5 ng of total RNA was separated on total RNA pico chips on Agilent 2100 Bioanalyzer. B: Scatter plots comparing the normalised read count for each annotated gene in GRCh38 in poly(A)+ RNA-seq libraries derived from unprocessed and from glyoxal fixed, permeabilised and stained cells. Two biological replicates were sequenced for each condition and averaged, read counts were normalised to the total number of reads in each library. Data is shown for both COLO205 and MCF-7 cells. C: Flow cytometry density plots for MCF-7 cells labelled with anti-CCNB1 primary antibody and donkey Alexa Fluor-488 conjugated secondary antibody and sorted using a BD FACSAria III sorter. Intact cells (shown within the elliptical gate) were distinguished from off-scale events and cell debris using forward scatter (FSC) and side scatter (SSC) measurements (panel 1). Doublets were excluded from the gated cells by a 2-step gating strategy with pulse height (H)

Cell line identity was validated based on RNA-seq data generated in this work

Add 1 ml of 1% BSA in PBS with 1:100 RNasin Plus and centrifuge at 2000 x *g* for 3 minutes at 4ÊC. Discard the

S2 Table. Genes differentially expressed between CCNB1 negative and positive cells. Genes differentially expressed between RNAseq libraries of CCNB1 ±ve and +ve MCF7 cells sorted by flow cytometry. Genes identified by DESeq2, $p < 0.05$.
(XLSX)

S3 Table. Gene ontology analysis of genes differentially expressed in CCNB1 positive cells. Gene Ontology enrichment analysis for genes listed in S2 Table was performed using GOrilla with an FDR cutoff 0.05.
(XLSX)

S4 Table. Primers used for RT-qPCR analysis.
(XLSX)

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