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ORIGINAL RESEARCH PAPER

A universal fixation method based on quaternary ammonium salts (RNA*later*) for omics-technologies: *Saccharomyces cerevisiae* as a case study

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Abstract Genomics, transcriptomics, proteomics and fluxomics are powerful omics-technologies that play a major role in today's research. For each of these techniques good sample quality is crucial. Major factors contributing to the quality of a sample is the actual sampling procedure itself and the way the sample is stored directly after sampling. It has already been described that RNA*later* can be used to store tissues and cells in a way that the RNA quality and quantity are preserved. In this paper, we demonstrate that quaternary ammonium salts (RNA*later*) are also suitable to preserve and store samples from *Saccharomyces cerevisiae* for later use with the four major omics-technologies. Moreover, it is shown that RNA*later* also preserves the cell morphology and the potential to recover growth, permitting microscopic analysis and yeast cell culturing at a later stage.

Keywords Fluxomics · Genomics · Omics · RNA*later* · *Saccharomyces cerevisiae* · Transcriptomics

Introduction

Room temperature immersion of fresh tissue samples in aqueous sulfate salt solutions (such as ammonium sulfate) at controlled pH precipitates degenerative RNAses (Allewell and Sama 1974) and other solubilized proteins, thereby preserving the tissue with intact RNA (Lader 2001). RNA*later* (Ambion, Applied Biosystems) is a product that is based on quaternary ammonium salts and has been used as an aqueous tissue storage reagent that stabilizes and protects cellular RNA in intact, unfrozen animal and plant tissue samples, tissue culture cells, blood and plasma, yeast cells and bacteria (Barrett et al. 2002; Dekairelle et al. 2007; Medeiros et al. 2003; Mutter et al. 2004; Williams 2010). It eliminates the need to process

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tissue samples immediately or to freeze samples in liquid N_2 for later processing. A disadvantage of frozen storage is that homogenization of frozen tissue must be accomplished rapidly to avoid the rapid RNA degeneration that occurs during thawing of a previously frozen sample. Using RNA*later*, samples can be stored for some time at ambient temperature, which allows sample collection outside the lab, such as out in the field (e.g. Van den Broeck et al. 2011), during marine research (Ottesen et al. 2011), microgravity research (Hammond et al. 2005; Kittang et al. 2010) or in the operation room (e.g. Mutter et al. 2004). Tissue pieces can be harvested and submerged in RNA*later* for storage without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation. RNA*later* preserved cells and tissues are suitable starting points for RNA quantification by quantitative RT-PCR (Bachoon et al. 2001) and gene expression microarray hybridization (transcriptomics) (Mutter et al. 2004).

Other RNA stabilization reagents are SUPERase•In RNase inhibitor (Ambion) and RNAprotect (Qiagen). The former product inhibits specifically RNases, whereas the latter has been introduced to stabilize bacterial RNA (Sirsat et al. 2011). RNAprotect salivary reagent has been successfully used for the stabilization of salivary RNA at room temperature (Park et al. 2006). Recently, it has been shown that RNA*later* can also be used for other applications than the stabilization of RNA. It has been used to preserve DNA (Michaud and Foran 2011) and mosquitoes for subsequent scanning and analysis by near-infrared spectroscopy (NIRS) (Sikulu et al. 2011).

Here, we show that the use of RNA*later* for RNA stabilization can be extended to the preservation of samples for various omics-technologies, i.e. transcriptomics, genomics, proteomics, and fluxomics. As a model system, the yeast, *Saccharomyces cerevisiae,* was selected since it was used in a microgravity experiment where cells where grown and fixed with RNA*later* in the International Space Station (Van Mulders et al. 2011). Additionally, we positively evaluated if RNA*later* can also be used to fix and preserve cells for further morphological and ultrastructural microscopic examination. Our findings also show that this fixation process is non-toxic and cells can be recovered and grown again after RNA*later* fixation and stowage.

Materials and methods

Cell culture

Saccharomyces cerevisiae strain BY4742 MAT α WT (S288C background) (Brachmann et al. 1998) was used. The following cell culture conditions were used, unless otherwise mentioned. For the liquid cultures, 1 ml YPD (10 g yeast extract/l, 20 g peptone/l, 40 g glucose/l) was inoculated with 10^7 cells in 1.5 ml screw cap tubes. After 40 h at 28 °C, the culture was centrifuged (5 min at 1,000×*g*) and supernatant was removed before addition of RNA*later* (1 ml added to the harvested cells) or flash-freezing (see below). For the cell cultures on agar, a 12-well multiplate (Falcon) was filled with 2 % agar YPD (10 g yeast extract/l, 20 g peptone/l, 20 g glucose/l, 20 g agar/l). From a cell suspension of 10^7 cells/ml, 1 µl was inoculated on the agar to initiate single colony growth. After 5 days of growth at 28 °C cells from the colony were collected by scraping and subsequently suspended in 1 ml RNA*later* or flash-frozen (see below).

Yeast cell preservation

To preserve the yeast cells for analysis at a later time, cells were treated in two different ways. Cells were flash-frozen by immersing them in liquid N_2 for a few seconds followed by storage at −80 °C for later use.

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Alternatively, cells were immersed in 1 ml RNA*later* solution (Ambion) and stored for 24 h at 4 °C. Subsequently, the RNA*later* cell suspension was centrifuged (5 min at $\sim 800\times g$) and the supernatant was discarded after which the cell pellet was stored at −80 °C for later use.

RNA extraction

For RNA extraction, an adapted version of the classical TRIzol (Invitrogen) method was used. The cell pellet was resuspended in 500 µl TRIzol reagent to which 500 µl of sterile RNase free glass beads (VWR, diam. 0.65–0.9 mm) were added. Cells were homogenized in a FastPrep homogenizer (MP Biomedicals) for 20 s at 6 m/s and afterwards immediately cooled on ice. Subsequently, 500 µl TRIzol reagent was added and was left to incubate for 5 min at room temperature (RT). Next, the suspension was centrifuged for 15 min at $\sim 16{,}000\times g$ at 4 °C and the TRIzol suspension was transferred to a clean Eppendorf tube. 200 µl chloroform was added and was mixed by vortexing at maximum speed for 10 s. The suspension was incubated for 3 min at RT and centrifuged for 15 min at $\sim 16{,}000\times g$ at 4 °C. Subsequently, the upper aqueous phase was transferred to a clean Eppendorf tube (the inter phase and bottom TRIzol phase was stored at −20 °C for later use) and 500 µl 2-propanol was added and mixed by inversion. Samples were stored overnight at −20 °C. Next, samples were centrifuged 10 min at $\sim 16{,}000\times g$ at 4 °C and the supernatant was carefully removed. The RNA pellet was washed twice with 1 ml 75 % (v/v) ethanol and finally dissolved in 100 µl RNase-free water. RNA samples were cleaned up using the RNeasy mini kit (Qiagen) including an on column DNase digestion according to the manufacturer's protocol. RNA concentration and purity were determined spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies) and RNA integrity was assessed using a Bioanalyzer 2100 (Agilent).

DNA extraction

DNA was extracted from the remaining inter and organic phase after RNA extraction. 500 µl back-extraction buffer [4 M guanidine thiocyanate, 50 mM sodium citrate, and 1 M Tris (free base)] was added to the sample and the sample was vortexed at maximum speed for 15 s. Samples were incubated for 10 min at RT and subsequently centrifuged for 4 min at $\sim 16{,}000\times g$ at 4 °C. The upper aqueous phase was transferred to a clean Eppendorf tube and 400 µl 2-propanol was added followed by mixing by inversion. Samples were left for 10 min at RT and subsequently centrifuged for 10 min at $\sim 16{,}000\times g$ at 4 °C. The supernatant was removed and the DNA pellet was washed twice with 75 % (v/v) ethanol. Finally the DNA pellet was dissolved in 30 µl milliQ water. DNA concentration and purity were determined spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies).

Microarray analysis

Per sample, 1 µg total RNA, spiked with 10 viral polyA transcript controls (Agilent), was converted to double-stranded cDNA in a reverse transcription reaction. Subsequently, the sample was converted to antisense cRNA, amplified and labeled with cyanine 3-CTP (Cy3) or cyanine 5-CTP (Cy5) in an in vitro transcription reaction according to the manufacturer's protocol (Agilent). A mixture of purified and labeled cRNA (Cy3 label: 5 pmol; Cy5 label: 3.5 pmol) was hybridized on microarrays printed with the Agilent SurePrint technology followed by washing, according to the manufacturer's procedures. The content of the arrays was exactly identical to the Agilent yeast (V2) gene expression microarray (product number: G4813A, design ID: 016322), but supplemented with probes tiling the complete transcript sequences of the flocculation genes *FLO1*, *FLO5*, *FLO9, FLO10*, *FLO11,* and the transcription factor gene *FLO8*. To assess the raw probe signal intensities, arrays were scanned using the Agilent DNA microarray scanner with surescan high-resolution technology and probe signals were quantified using Agilent's feature extraction software (version 9.5.1.1).

qRT-PCR analysis

The level of *FLO1* gene expression in RNA*later*-preserved cells and in flash-frozen cells were compared using qRT-PCR. For each sample, 1 µg total RNA was subjected to reverse transcription (RT) using an RT system (A3500; Promega). Concentrations were determined, and samples were diluted to obtain a concentration of 100 ng/µl. The 25 µl PCR mixture

consisted of 12.5 µl Power SYBR Green (Applied Biosystems) and 1.25 ml of each primer (500 nM). 5 µl cDNA (100 ng/µl) was added to each reaction mixture. The two-step PCR program on the ABI Prism 7500 instrument (Applied Biosystems) consisted of an initial denaturation for 10 min at 95 °C and amplification using 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The levels of expression of the different genes were all normalized with respect to the levels of *ACT1* expression. A standard curve of each gene was constructed with genomic DNA. The expression levels were analyzed with SDS software (Applied Biosystems). The PCR primers were all designed with the PRIMER EXPRESS software (Applied Biosystems) according to the Applied Biosystems guidelines. The primer sequences used for qRT-PCR analyses are *FLO1*_qRTPCR_FW (5′-TAGCTGCTGAGACGATTACCAA-3′) and *FLO1*_qRTPCR_RV (5′-GCGTGATTAGATCTTGAAAGCGAA-3′), and *ACT1*_qRTPRC_FW (5′-CGTCTGGATTGGTGGTTCTA-3′) and *ACT1*_qRTPCR_RV (5′-GTGGTGAACGATAGATGGAC-3′).

PCR analysis

To validate the use of gDNA derived from RNA*later*-treated cells, a standard PCR was performed using primers *FLO1*_FW (5′-ATGACAATGCCTCATCGC-3′) and *FLO1*_RV (5′-CTTCCACCCCATGGCTTGATACCGTC-3′) to detect a 596 bp fragment within the *FLO1* gene. Therefore, *Taq* DNA polymerase (Roche) was used according to the manufacturer's protocol. Initial denaturation and enzyme activation at 94 °C, was followed by 25 cycles consisting of a denaturation step during 15 s at 94 °C, a primer hybridization step of 1 min at 58 °C and an elongation step of 1.5 min at 72 °C.

Protein extraction

The RNA*later*-preserved cell pellets were first washed with GTE buffer [50 mM Tris/HCl pH 7.4, 10 % (v/v) glycerol, 1 mM EDTA], and subsequently, proteins were extracted as follows. Yeast cells were resuspended in 500 µl lysis buffer [7 M urea, 2 M thiourea, 20 g CHAPS/l, 10 g dithiothreitol (DTT)/l, 0.8 % (v/v) citric acid, protease inhibitor cocktail (Roche)], glass beads (450–600 µm) were added and cells were lyzed by vortexing 5 times for 30 min. After centrifugation (20 min at 10,000×*g*, 4 °C) the supernatant containing the soluble proteins was transferred to a new Eppendorf tube. Protein yield and quality were determined using respectively the Bradford assay and SDS-PAGE.

For protein quality control, a SDS-PAGE gel was run where 10 µg protein was dissolved in Laemmli buffer (Laemmli 1970) and loaded onto a 12 % discontinuous acrylamide SDS-PAGE gel. Electrophoresis was performed for 10 min at 100 and 150 V until the bromophenol blue front migrated off the gel. The SDS-PAGE gels were stained with Coomassie-G250 and scanned with a GS-800 scanner (Biorad).

Mass spectrometry

Some protein bands were excised manually for mass spectrometric analysis. An in-gel digestion protocol was performed. First, the gel spots were washed twice with 50 % (v/v) acetonitrile/200 mM NH_4HCO_3 for 20 min at 30 °C. Subsequently, the spots were dried, a 1/50 dilution of 0.1 µg trypsin/µl was added, and after 45 min on ice 50 mM NH_4HCO_3 was added until the spots were completely submerged. Digestion was performed by overnight incubation at 37 °C. Extraction of the peptides from the gel pieces was achieved by adding twice 60 % (v/v) acetonitrile/0.1 % (v/v) formic acid to the gel spots. The final volume of extraction buffer was dried in a Speedvac, and finally the peptides were dissolved in 8 µl 0.1 % (v/v) formic acid. After the peptide extraction, 0.5 µl peptide mixture was spotted on a stainless steel MALDI target plate and covered with 0.5 µl α-cyano-4-hydroxy cinnamic acid matrix (7 mg/ml in 50 % (v/v) acetonitrile, 0.1 % (v/v) trifluoroacetic acid, 1 mM ammonium citrate). Identification of the proteins was obtained by measuring the peptide mass fingerprint (PMF) on a MALDI-TOF/TOF MS system (4700 proteomics analyzer, Applied Biosystems) in MS mode. The sequence of at least three peptides was verified in MS/MS mode. The obtained spectra were searched against a *S. cerevisiae* database, downloaded from NCBI, with an "in-house" Mascot platform using the Mascot algorithm (Matrixscience, London, UK). The spectra were searched with a tolerance of 100 ppm for the peptide mass and of 0.5 Da for the MS/MS data, with carbamidomethylation (Cys) and oxidation (Met) as variable modification parameters.

Fluxomics

Yeast cells were grown in minimal medium (Verduyn et al. 1992) containing 7.5 g $(NH_4)_2SO_4$/l, 14.4 g KH_2PO_4/l, 0.5 g $MgSO_4 \cdot 7H_2O$/l and 20 g glucose/l of which 50 % was 1-^{13}C-labelled at 150 rpm and 30 °C for 14 h to an OD_{600} of ~5.0 (late active-growth phase). Three samples of 30 ml culture were harvested by centrifugation (5 min at 4 °C and 5,000×*g*) and the supernatant was removed. The cell pellet in the first sample (no. 1) was washed once with water and then stored at −20 °C. The pellet in sample no. 2 was resuspended in 1 ml supernatant and 1 ml RNA*later*, incubated at 30 °C for 11 h, centrifuged for 5 min at 4 °C and 5,000×*g*, washed once with water and then stored at −20 °C. The cell pellet in sample no. 3 was resuspended in 2 ml RNA*later* and treated as described for sample no. 2.

Hydrolysis of the samples was performed as described before (Gombert et al. 2001). Subsequently, each sample was divided in two, derivatized using ethylchloroformate (ECF) and (*N*,*N*)-dimethylformamide dimethylacetal (DMFDMA) respectively, and subjected to GC–MS analysis.

Microscopic bud scar and actin analysis

To evaluate the effectiveness of RNA*later* treatment in fixing cells for microscopic morphology analysis, bud scar profiling and actin analysis were selected as analysis types. Staining of bud scars was performed according to the method described by Lord et al. (2002). Briefly, cells were fixed by 3.7 % (v/v) formaldehyde and washed twice with PBS. Chitin, especially present in the bud scars, was visualized by treating with Calcofluor white (1 mg/ml) (Sigma) (Pringle 1991). Again, cells were washed twice with PBS. Cells were resuspended in 100 µl PBS with 1 % Triton and supplemented with 40 µl rhodamine–phalloidin (Invitrogen) and incubated for 60 min. Again, cells were washed twice with PBS. According to the amount of cells, 50 µl *p*-phenylemidiamine was added to the cells to reduce photobleaching.

To immobilize the cells, the microscope slides were immersed in concanavalin A/PBS (0.1 mg/ml) for 15 min at room temperature. Five microliter sample was mounted onto the slide and the stained cells were observed by epifluorescence microscopy (Olympus BX61 TRF), using the 100× objective lens and appropriate filter sets for Calcofluor (Sigma-Aldrich) emission and rhodamine–phalloidin emission wavelengths.

Yeast growth and fermentation after fixation with RNAlater

To evaluate the possibility of re-culturing yeast cells after RNA*later* treatment, yeast growth in liquid YPD growth medium and on semi-solid YPD agar medium was examined after storage at −80 °C (reference) and for cells after RNA*later* treatment.

Saccharomyces cerevisiae was initially cultured from an −80 °C stock on YPD agar. A single colony was inoculated in 5 ml YPD at 22 °C for 24 h and further used to inoculate 50 ml YPD (with 40 g glucose/l), which was inoculated at ~10^6 cells/ml during 48 h at 22 °C. For the reference sample, a 1 ml sample was centrifuged and the pellet was flash-frozen in liquid N_2 and stored at −80 °C. For the RNA*later*-treated sample (see above), a 1 ml sample was centrifuged and the pellet was resuspended in 1 ml RNA*later* during 4 days at 4 °C for fixation. After this fixation period, 10 µl cell suspension was inoculated on YPD agar and incubated at 23 °C during 5 days. A single colony was flash-frozen in liquid N_2 and further stored at −80 °C.

Next, to assess possible growth and performance differences between the reference and the RNA*later*-treated cells, these two samples were cultured from the −80 °C stock on YPD agar. A single colony was inoculated in 5 ml YPD at 20 °C for 24 h and further used to inoculate 50 ml YPD (with 40 g glucose/l) at ~10^6 cells/ml for 24 h at 20 °C. From this yeast suspension, colony growth was followed on YPD solid medium agar and cell density in YPD liquid medium. At different times, the cell concentration of the liquid cultivation was determined by measuring the OD_{600}. [Samples were centrifuged (5 min at 1,000×*g*), diluted in PBS to give an $OD_{600} < 0.8$]. The glucose concentration in the filtered medium was determined using a glucose oxidase peroxidase enzyme kit.

Inoculation on YPD agar was performed by 1 µl drops from a yeast suspension (~10^6 cells/ml) in isotonic saline. For the liquid medium, YPD (4.5 g glucose/l) was inoculated at 10 × 10^6 cells/ml. Pictures of the top view of the colonies on YPD agar were taken at different time points during 12 days and the

colony diameter was measured using ImageJ software (Abramoff et al. 2004).

Results and discussion

Transcriptomics

RNA extracted from yeast cells, which had been cultivated in liquid growth medium and preserved in RNA*later,* was compared to RNA extracted from flash-frozen yeast cells. The latter is considered to be the 'golden' standard, as it is often advised to flash-freeze samples to preserve the RNA quality for later transcriptomics research. After sample storage, RNA was extracted at a later time point. No significant difference could be observed concerning RNA purity (Nanodrop A_{260}/A_{280} and A_{260}/A_{230} ratios), integrity (RIN values), and quantity between the RNA*later* preserved samples and the flash-frozen samples (see Table 1; Fig. 1). Nanodrop A_{260}/A_{280} and A_{260}/A_{230} were all above 2.2 and RIN values where on average 6.7 (SD = 0.46) and 6.30 (SD = 0) for flash-frozen and RNA*later* preserved samples, respectively. The Nanodrop results indicated that the samples were pure and not contaminated with any proteins or organic salts, as both ratios were well above the generally accepted cut-off of 1.8. In general, RNA of good integrity has RIN values larger than 7–8. Although the RIN values here are somewhat lower, RIN values are similar for flash-frozen and RNA*later* preserved samples. Furthermore, the RIN values for *S. cerevisiae* total RNA might not be represenative, as the RIN-software algorithm (Schroeder et al. 2006) was trained on eukaryotic total RNA form human, mouse, and rat. Moreover, samples were perfectly suitable for transcriptomics analyses using real-time PCR and microarray analysis (data not shown).

Genomics

DNA was extracted from the remaining organic and inter phase after RNA extraction of RNA*later* preserved samples of three agar and three liquid cultures. Table 2 displays the Nanodrop results. DNA was of good purity with A_{260}/A_{280} ratio's larger than 1.8 for the majority of the samples. Only one sample had a slightly smaller ratio, being 1.73, which is still an acceptable value. A_{260}/A_{230} ratios were ranging from 0.91 to 2.45 for the agar samples, and from 0.33 to 0.8 for the liquid samples. The lower values for the liquid samples are most likely caused by the lower concentrations that were measured (21.5–37.2 ng/µl), resulting in an artificial increase of the A_{260}/A_{230} ratio. Yields ranged from 3.1 to 8.97 µg for the agar culture samples, and 0.65–1.12 µg for the liquid culture samples. These amounts are sufficient for the conduction of DNA experiments such as PCR, array CGH, or Next Generation sequencing for which in most applications an amount of 500 ng genomic DNA can be used as input. PCR reactions were successfully performed for three samples of each culture condition (agar and liquid cultivation), as illustrated in Fig. 2.

Proteomics

Proteomic analysis was performed in parallel to the transcriptomic and genomic analyses. RNA*later* preserved cells were compared to cells, which were flash frozen. After protein extraction, the obtained yields were compared. For both yeast cells grown in culture and grown on agar, the yield was higher in RNA*later* preserved cells (see Fig. 3). The protein quality was assessed using SDS-PAGE, and there is no clear difference in protein patterns when preserved in RNA*later* indicating that in both situations the proteins were not degraded (Fig. 4). Moreover, it

Table 1 Nanodrop and Bioanalyzer measurement results of RNA from flash-frozen and RNA*later*-preserved liquid cell culture samples

Sample ID	Preservation	Yield (µg)	Concentration (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}	RIN value
Ff 1	Flash-frozen	3.8	128	2.24	2.31	6.20
Ff 2	Flash-frozen	6.1	202	2.22	2.37	7.10
Ff 3	Flash-frozen	4.7	156	2.24	2.43	6.80
Rl 1	RNA*later*	2.6	87	2.23	2.21	6.30
Rl 2	RNA*later*	3.3	111	2.24	2.25	6.30
Rl 3	RNA*later*	4.0	134	2.23	2.29	6.30

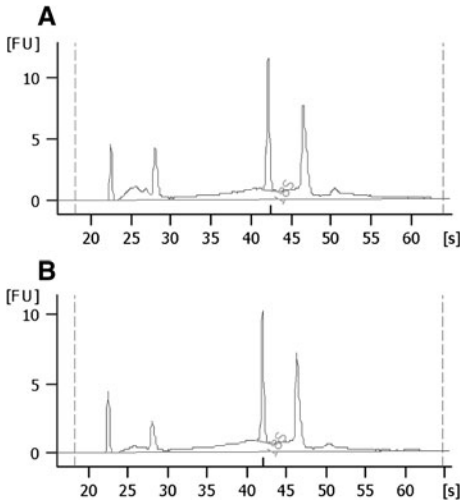

Fig. 1 Bioanalyzer results providing an indication of the integrity of the RNA samples. The RNA integrity of both **a** flash-frozen (sample Ff 1 from Table 1) and **b** RNA*later* (sample Rl 1 from Table 1) preserved samples is equally well. Results of only two samples are shown, as the results of the other samples are similar

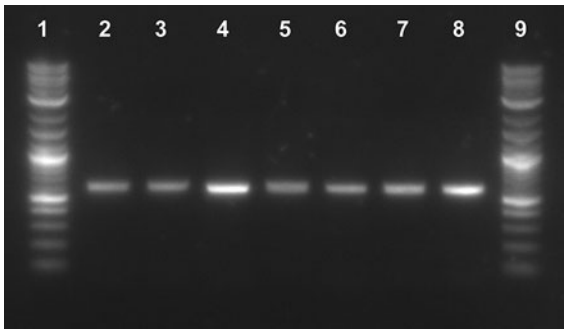

Fig. 2 PCR analysis for the *FLO1* gene on flash-frozen samples (*lane 2*), RNA*later* preserved samples of agar (*lanes 3–5*) and liquid (*lanes 6–8*) yeast cell cultures. *Lanes 1* and *9* display a 2-log DNA ladder (New England Biolabs). PCR resulted in a band of 596 bases in size

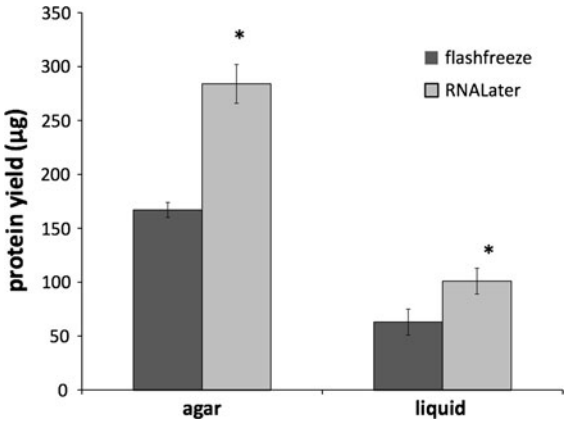

Fig. 3 Yield of protein extraction of flash-frozen and RNA*later* preserved agar and liquid culture samples. Data points are mean values of four replicates. *Error bars* correspond to standard deviations; *asterisks* indicate statistically significant differences between flash-freezing and RNA*later* treatment (* $p < 005$)

was possible to perform successful mass spectrometric (MS) protein identification on the protein extracts from the RNA*later* preserved cells. This indicates that there is no interference of the RNA*later* with MS analysis in contrast to other fixating methods such as formaldehyde that forms protein cross-linking, which can interfere with MS protein identification (Fraenkel-Conrat and Olcott 1948; Richert et al. 2004).

Fluxomics

For metabolic flux analysis (MFA), cells are usually grown on a ^{13}C-labelled substrate, e.g. glucose. A typical approach is the analysis of the labeling pattern of proteinogenic amino acids. For this purpose, cells are normally harvested by centrifugation or filtration and then stored at −20 or −80 °C (Christen and Sauer 2011; Gombert et al. 2001). To test whether the preservation with RNA*later* of cells grown in presence of ^{13}C-labelled glucose had any influence on their

Table 2 Nanodrop measurement results of DNA extractions from RNA*later* preserved yeast cells

Sample ID	Culture type	Yield (µg)	Concentration (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}
A 1	Agar	9.0	299	2.08	1.91
A 2	Agar	5.1	170	1.94	2.45
A 3	Agar	3.1	103	2.08	0.91
L 1	Liquid	1.0	32	1.80	0.64
L 2	Liquid	0.7	22	1.73	0.80
L 3	Liquid	1.1	37	1.89	0.33

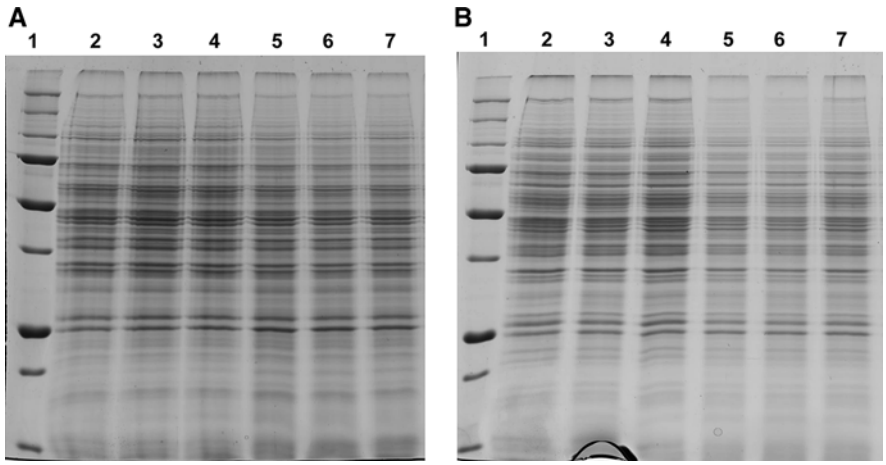

Fig. 4 SDS-PAGE of proteins extracted from cells grown on agar (**a**) and in liquid (**b**) culture. The same samples were loaded in both gels shown in images (**a**) and (**b**). *Lane 1*: Precision plus protein unstained standards (BioRad), *lanes 2–4*: flash-frozen samples, *lanes 5–7*: RNA*Later* preserved samples

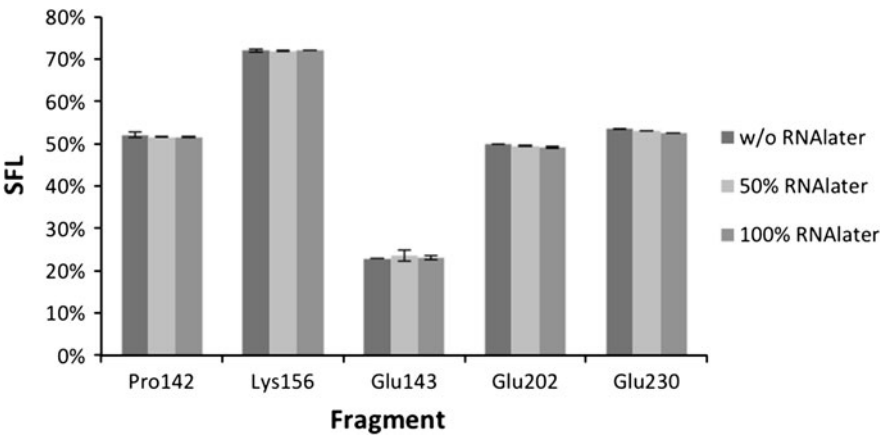

Fig. 5 Summed fractional labeling (SFL) of selected amino acids fragments in cells grown on ^{13}C-labeled glucose and incubated in different concentrations of RNA*later*. Data points are mean values of 2 replicates; *error bars* correspond to standard deviations

amino acid labeling pattern, RNA*later* preserved cells were compared with cells stored immediately at −20 °C. Figure 5 shows the summed fractional labeling of the fragments derived from proline, lysine, and glutamate. There was no difference in the ^{13}C-labeling patterns of the samples stored at −20 °C compared to the samples preserved with 50 or 100 % RNA*later* solution. As the labeling pattern of these amino acids allows for flux quantification, it shows that RNA*later* can be used also for storing samples for fluxomics.

Microscopy

RNA*later* preserved cells were compared to flash-frozen cells. Figure 6 illustrates the microscopic results of bud-scar and actin staining on flash-frozen as well as on RNA*later* preserved cells from liquid cultures. Bud-scar and actin analysis could be performed equally well for cells stored in both conditions.

Yeast growth and fermentation after fixation with RNAlater

It was also demonstrated that cells maintained the ability to be re-cultured after preservation with RNA*later* as they were able to grow normally again under standard culture conditions, as illustrated by cell concentration, glucose consumption, and colony diameter measurements (see Fig. 7).

Conclusion

RNA*later* is an excellent storage agent for yeast cells and, most likely, also for other cell types and tissues, in

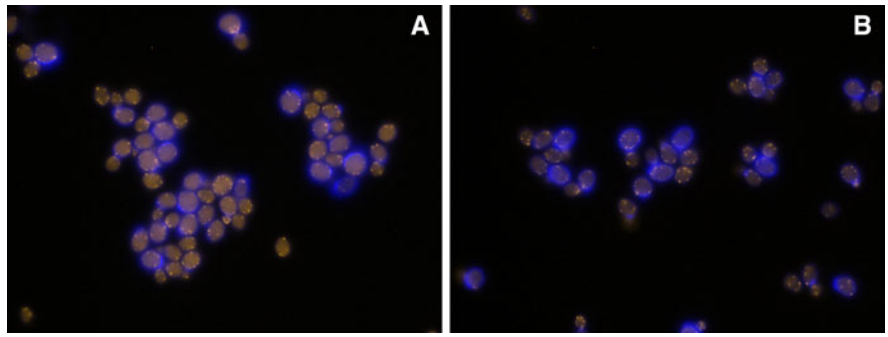

Fig. 6 Microscopic bud scar and actin staining of flash-frozen (**a**) and RNA*later* (**b**) preserved liquid culture samples. Bud scar (*blue*) and actin (*orange*) visualization with calcofluor and rhodamine–phalloidin, respectively

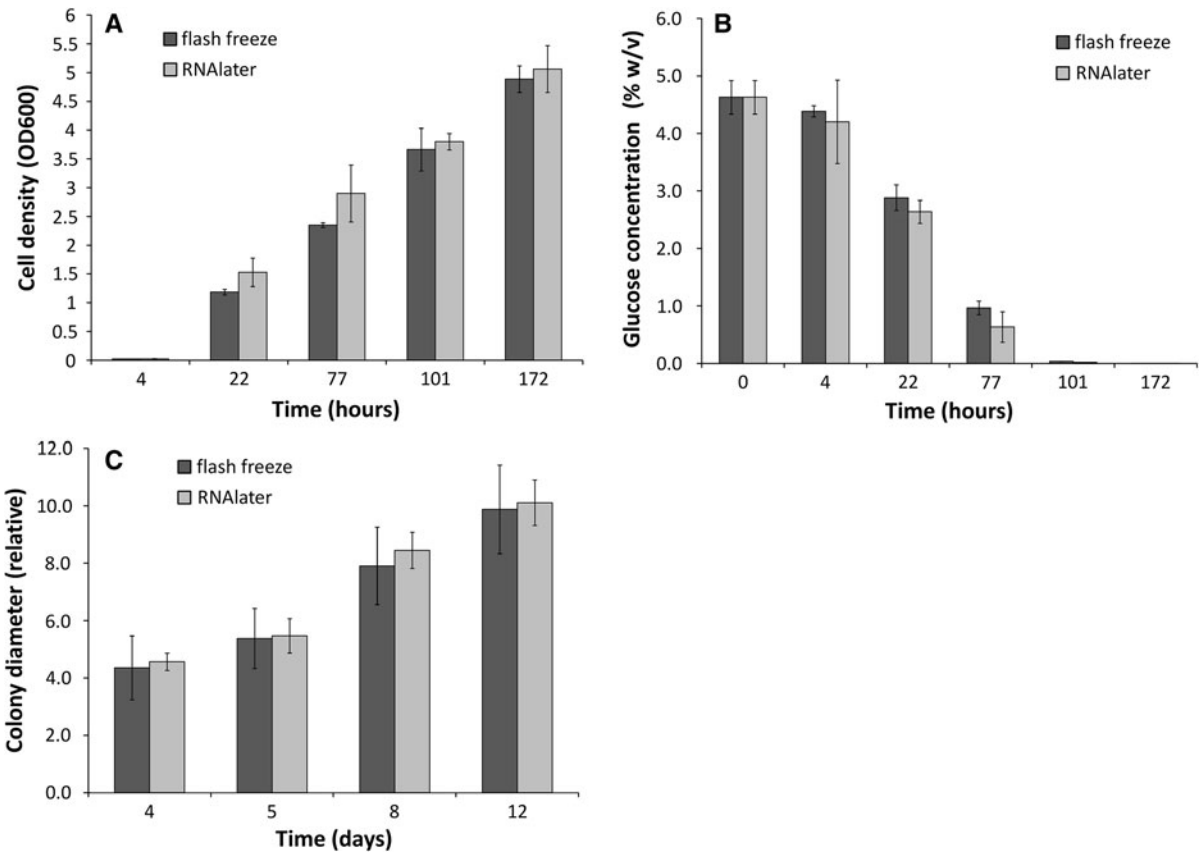

Fig. 7 Results of the density (**a**) and glucose concentration (**b**) of a liquid cell culture, inoculated with cells which were previously exposed to a flash-freeze storage step or a RNA*later* storage step. Evolution of the colony diameter of cells that were previously exposed to a flash-freeze storage step or a RNA*later* storage step (**c**). Data points are mean values of two replicates; *error bars* correspond to the standard deviation

order to preserve the samples for later subjection to the major omics-techniques and microscopic analyses. Moreover, yeast cells remained viable after preservation with RNA*later*, making it a good alternative when other storage methods, often using liquid N_2, are not available. For these reasons, RNA*later* is in particular of interest for field experiments or experiments in special environments where flash-freezing is not evident.

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