Chapter 8

Biotin-Genomic Run-On (Bio-GRO): A High-Resolution Method for the Analysis of Nascent Transcription in Yeast

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Abstract

Transcription is a highly complex biological process, with extensive layers of regulation, some of which remain to be fully unveiled and understood. To be able to discern the particular contributions of the several transcription steps it is crucial to understand RNA polymerase dynamics and regulation throughout the transcription cycle. Here we describe a new nonradioactive run-on based method that maps elongating RNA polymerases along the genome. In contrast with alternative methodologies for the measurement of nascent transcription, the BioGRO method is designed to minimize technical noise that arises from two of the most common sources that affect this type of strategies: contamination with mature RNA and amplification-based technical biasing. The method is strand-specific, compatible with commercial microarrays, and has been successfully applied to both yeasts *Saccharomyces cerevisiae* and *Candida albicans*. BioGRO profiling provides powerful insights not only into the biogenesis and regulation of canonical gene transcription but also into the noncoding and antisense transcriptomes.

Key words Nascent transcription, RNA polymerase II, RNA polymerase II I, Yeast, *Saccharomyces cerevisiae*, *Candidaalbicans*, Nascent RNA

1 Introduction

Transcription is the first step in the gene expression process. It is also believed to be the most regulated step in eukaryotes. Because of that, the study of eukaryotic transcription is one of the main topics of molecular biology. Many in vivo and in vitro procedures have been developed to study the transcription cycle of eukaryotic RNA polymerases (reviewed in 1, 2) Recently, with the advent of genomic methodologies it has been possible to study the particular features of every single gene. At the same time, doing average profiling for all genes allows to determine the real properties of a typical gene instead of extrapolating those of a particular experimental example to the whole genome [3].

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To this end, some high-resolution techniques for the study of nascent transcription have been established [4–6]. Each technique has particular features that reveal different aspects of the transcription process (reviewed in 3, 7). Chromatin immunoprecipitation (ChIP) detects all RNA pol, active or not. However, it can differentiate between different RNA pol species, or carboxy-terminal (CTD) phosphorylated forms of RNA pol II, or even elongating complexes with different composition by using specific antibodies [7]. Techniques that detect nascent RNA (nRNA) only detect elongating RNA pol, allowing their mapping at high resolution [4–6]. They are, however, unable to distinguish between active RNA pol II molecules and those that are backtracked but still retaining a bound RNA molecule. Genomic run-on approaches (GRO, 8, 9), however, only detect active elongating RNA pol I, II, and III molecules.

Variants of GRO have been published by other laboratories working in yeast or higher eukaryotes [10–12]. All those methods use next generation sequencing for the analysis of purified nRNA. Purification of the very rare nRNA requires its labeling with a precursor, such as BrUTP or Biotin-UTP. Because of the small proportion of nRNA in the cell, contamination with mature RNA is an important concern. The presence of such contaminant may obscure the conclusions drawn from those methods. However, by hybridizing in vivo-biotinylated RNAs directly onto the arrays, the risk of mature RNA contamination and of any technical noise derived from amplification may be bypassed. This improvement could in turn help to draw more powerful biological insights when analyzing the results.

We have taken profit of the fact that Affymetrix arrays are based on detecting biotin labeled nucleic acids to hybridize our in vivo-biotinylated RNAs directly onto them. We call this protocol Biotin-GRO or BioGRO (Fig. 1). In this way, small amounts of contaminant mature RNA (rRNA, mRNA or any other) become unimportant because they do not fluoresce upon laser scanning. This is the same situation observed in the classic radioactive run-on protocol [9], in which a large amount of nonradioactive mature RNA neither blocks nRNA hybridization nor interferes with its detection [13]. We observed, however, that in the conditions of Affymetrix hybridizations, the presence of the much more abundant non-labeled RNA severely reduces fluorescent signal. This is probably due to the high sample concentration, >100 times higher than for macroarrays. Therefore, with the aim of reducing the amount of contaminant RNA, we treated sarkosyl-permeabilized cells with RNase A. This treatment has been previously shown to destroy most of the preexisting RNA in mammalian cells without affecting run-on efficiency because of the protection offered by elongating RNA pol to their nRNA, known as RNA pol footprinting [14]. RNase A treatment, thus, allowed to eliminate most of

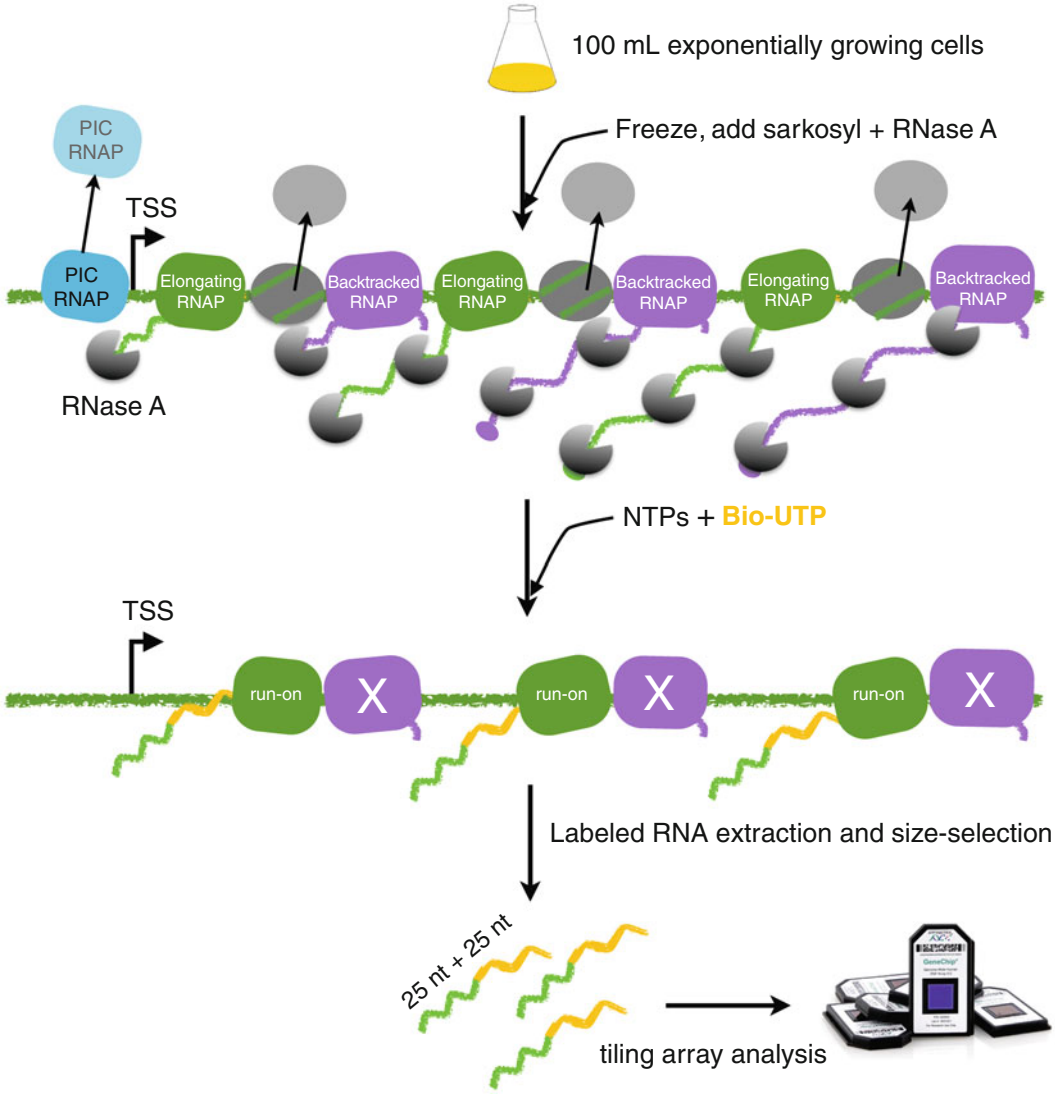

Fig. 1 Outline of the BioGRO method. The colors of the RNA polymerases (RNAP) represent different transcriptional states. Only active RNAPs (*green*) are elongation-competent during run-on. *Green* portions of nascent RNA molecules represent the footprints after RNase A digestion whereas *yellow* portions represent the run-on elongations

the mature RNA present in the cell and to trim the 5′ tail of nRNA giving a footprint of about 25 nt [14]. These molecules are then extended by around 25–30 nt during run-on, allowing the incorporation of some biotinylated uridine residues (*see* Fig. 1).

Here we describe a straightforward, strand-specific, high-resolution GRO technique for the model organisms *S. cerevisiae* and *C. albicans*, based on the use of a modified RNA precursor (biotin-UTP), and tiling microarrays. Overall, this method allows for the analysis of nRNA without any interference of mature RNA molecules for a large set of genes (*see* Figs. 2 and 3).

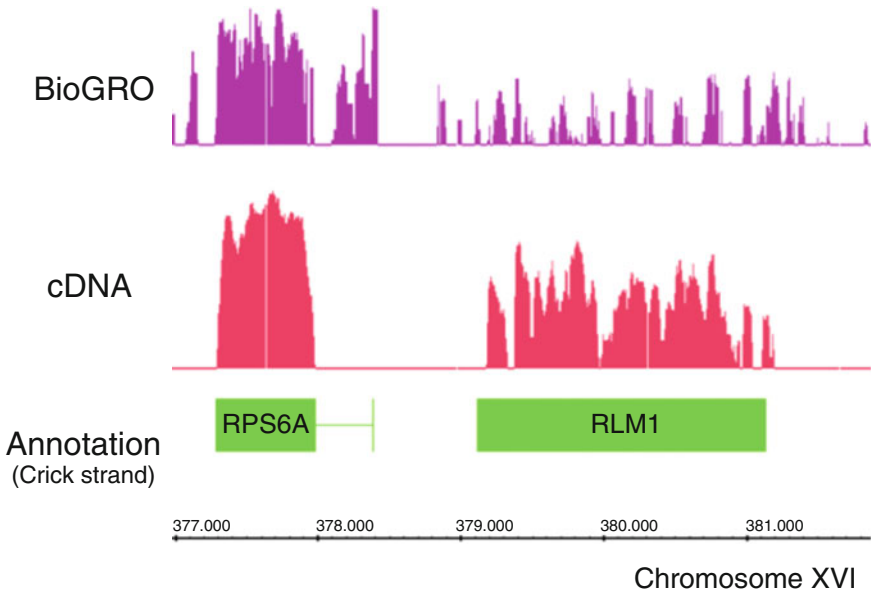

Fig. 2 Example of BioGRO signal along individual genome regions. The intronic region of gene RPS6A shows a BioGRO signal that is not seen in the same region in a mature mRNA hybridization

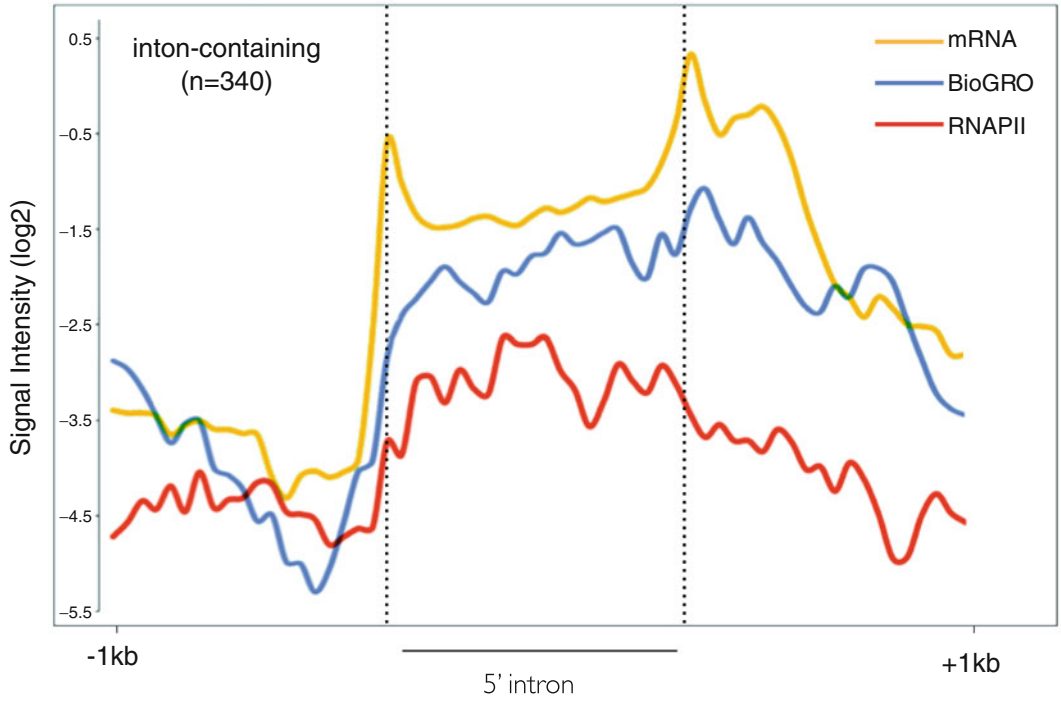

Fig. 3 BioGRO and RNA pol II ChIP measure nascent transcription, genome-wide. Average metagene profile of the 5′ intron region (in arbitrary units, normalized to 1 kb; flanking regions in real units) of 340 intron-containing genes. In contrast with the signal drop shown by the mRNA profile (*orange trace*), the generally flat density profile of both BioGRO (*blue trace*) and RNA pol II ChIP (*red trace*) data (provided by Sebastián Chávez, personal communication), argue in favor of their ability to capture elongating RNA pol II (and, thus, nRNA) and not fully processed, mature mRNAs. *Vertical dotted lines* mark exons–intron junctions

2 Materials

Precautions should be taken to minimize RNase contamination throughout all the protocol steps (*see* **Notes 1** and **2**).

2.1 Equipment

1. Low-speed table top centrifuge.
2. Refrigerated microcentrifuge.
3. Temperature-controlled orbital shaker.
4. DNA LoBind 1.5 mL Tubes (Eppendorf).
5. Eppendorf Thermomixer® Comfort Heating and Cooling Shaker.
6. NanoDrop ND1000 Spectrophotometer.
7. Affymetrix Hybridization and Wash Station and GeneChip® Scanner.
8. Thermoblock heater.
9. Savant SPD111V SpeedVac Concentrator (Thermo Scientific).
10. (Optional) GS Gene Linker UV Chamber (Bio-Rad).

2.2 Nascent RNA Biotinylation by Run-On

1. YPD medium: 1 % w/v, yeast extract, 2 % w/v, peptone, 2 % glucose. Store at room temperature (*see* **Note 3**).
2. 0.5 % w/v, l-laurylsarcosine (sarkosyl) in nuclease-free H_2O. Store at room temperature.
3. 2.5× transcription buffer: 50 mM Tris–HCl, pH 7.7, 50 mM KCl, 80 mM $MgCl_2$. Store at room temperature.
4. ACG mix (ATP, CTP, GTP, 10 mM each). Store frozen.
5. 0.1 M DTT. Store frozen.
6. Biotin-11-UTP (10 mM, Ambion). Store frozen.
7. Transcription mix: 120 µL of 2.5× Transcription buffer, 16 µL ACG mix, 6 µL 0.1 M DTT, and 20.25 µL of Bio-11-UTP. Prepare fresh.
8. RNaseOUT. Store frozen.
9. RNase A. Store at 4 °C.
10. 5 M Sodium acetate (pH 5.2). Store at room temperature.
11. 11.1 M Tris–HCl (pH 7.4). Store at room temperature.
12. Isopropanol. Store at room temperature.
13. Glycogen, for molecular biology. Store frozen.
14. DNase I, RNase-free. Store frozen.
15. Liquid nitrogen.
16. Proteinase K, recombinant, PCR grade. Store at 4 °C.
17. Ethanol, absolute.

18. Nuclease-free water, molecular biology grade.
19. MasterPure™ Yeast RNA Purification Kit (Epicentre).

2.3 Nascent RNA Size Selection

1. NucleoSpin® miRNA kit for small and large RNA species (Macherey-Nagel).
2. Ethanol, absolute.
3. Nuclease-free water, molecular biology grade.

2.4 Affymetrix Tiling Arrays

1. GeneChip® WT Terminal Labeling Kit (Affymetrix).
2. GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix).
3. GeneChip®*S. cerevisiae* Tiling 1.0R Array (Affymetrix).
4. GeneChip®*S. cerevisiae* Tiling Array Custom (Affymetrix).
5. GeneChip®*Candida* Custom Array (Affymetrix).

3 Methods

The method described here has been successfully applied to both *S. cerevisiae* [15] and *C. albicans* (unpublished results). However, this protocol focuses mainly in describing the BioGRO protocol for *S. cerevisiae*. In the case of *C. albicans*, and although the procedures are very similar, there are some variations which will be described in its own subheading. The major steps of this method are outlined in Fig. 1.

3.1 Preparation of DNase-Free RNase A

1. Dissolve RNase A at a concentration of 10 mg/mL in 0.01 M sodium acetate (pH 5.2).
2. Heat to 100 °C in a thermoblock for 15 min.
3. Allow it to cool down slowly to room temperature.
4. Adjust the pH by adding 0.1 volume of 1 M Tris–HCl (pH 7.4).
5. Dispense in aliquots and store at −20 °C.

3.2 BioGRO Method

1. Allow cells to grow in YPD (*see* **Note 3**) to the desired OD_{600} (typically 0.5–0.6).
2. For each sample, an aliquot of 100 mL is needed (corresponding to 12×10^8 cells, *see* **Note 4**).
3. Collect cells by centrifugation in two 50 mL falcon tubes at $4400 \times g$ for 2.5 min. From now onwards, both tubes are processed the same, in parallel, and the extracted RNA is pooled together at the end.
4. Decant the supernatant and submerge the pellet-containing tube in liquid nitrogen for flash freezing (*see* **Note 5**).

5. Transfer the frozen pellet to −20 °C. Keep the tube in the freezer for at least 3 h. This is a safe stopping point, as cells can be stored for longer periods (*see* **Note 6**).
6. Slowly thaw cells on ice and add 10 mL of a 0.5 % sarkosyl solution. Mix by inversion.
7. Pellet cells by centrifugation as in **step 3**, and discard the supernatant.
8. Resuspend cells in 3.2 mL of 0.5 % sarkosyl and add 32 μL of 10 mg/mL DNase-free RNase A. Mix by pipetting up and down several times.
9. Incubate cells with RNase A for 10 min at 30 °C in an orbital shaker to avoid sedimentation of cells at the bottom of the tube (*see* **Note 7**).
10. After 10 min, bring the volume up to 45 mL with sarkosyl 0.5 %. Mix vigorously by inversion to wash the cells and eliminate RNase A.
11. Recover cells by centrifugation as in **step 3**. Discard the supernatant.
12. Resuspend cells in 45 mL of 0.5 % sarkosyl. Shake vigorously by inversion and pellet the cells again.
13. Repeat previous step for a third and final wash.
14. Resuspend the pellet in 1 mL of 0.5 % sarkosyl and transfer cells to an Eppendorf tube (1.5 mL).
15. Recover cells by centrifugation at 5400 × *g* for 1 min in a microcentrifuge. Carefully remove the supernatant by pipetting and centrifuge again, if necessary, to eliminate any remaining sarkosyl.
16. Resuspend cells in 113.5 μL of nuclease-free water.
17. Add 5 μL of RNase inhibitor (RNaseOUT) to protect the integrity of nascent RNAs from any residual RNase that might be present after the washes. Mix by pipetting up and down several times. Keep cells on ice until needed.
18. Prepare the transcription mix: 120 μL of 2.5× transcription buffer, 6 μL 0.1 M DTT, 16 μL of ACG mix, and 20.25 μL of 10 mM Biotin-11-UTP (*see* **Notes 8** and **9**).
19. Pre-warm both cells and transcription mix at 30 °C for 5 min.
20. Add the transcription mix (162.25 μL) to the cell suspension and mix by pipetting.
21. Perform the run-on reaction by incubating the mix for 5 min at 30 °C in a thermomixer, with 550 rpm agitation (*see* **Note 10**).
22. Stop the reaction by adding 1 mL of ice-cold nuclease-free water to the tube. Snap cool and maintain on ice for 5 min.
23. Harvest cells by centrifugation for 1 min at 11,000 × *g* at 4 °C. Remove the supernatant (containing unincorporated nucleotides).

3.3 RNA Extraction and DNA Removal

RNA extraction was done with the MasterPure™ Yeast RNA Purification Kit, with some major modifications. Thus, and for clarity purposes, this section describes a continuous protocol that integrates our modifications with the kit manufacturer's instructions (*see* **Notes 11** and **12**).

1. Dilute 2.78 μL (50 μg) of Proteinase K into 300 μL of Extraction Reagent for RNA.
2. Add the mixture to the cell pellet from **step 23** of Subheading 3.2 and resuspend by pipetting up and down several times.
3. Incubate at 70 °C for 15 min in a thermomixer, with constant 600 rpm shaking. Additionally, vortex mix every 5 min to avoid cell deposition at the bottom of the tube.
4. Place the samples on ice for 3–5 min and add 175 μL of MPC Protein Precipitation Reagent. Vortex for 10 s.
5. Pellet the debris by centrifugation for 10 min at 4 °C at 12,000 × *g*.
6. Instead of a normal microcentrifuge tube, transfer the supernatant to a clean DNA LoBind Tube (*see* **Note 13**). Discard the pellet.
7. Add 500 μL of isopropanol and 10 μg of glycogen to the recovered supernatant (*see* **Note 14**). Mix by inversion 5–10 times.
8. Precipitate RNA overnight at –20 °C.
9. Pellet the RNA by centrifugation at 4 °C for 20 min at 12,000 × *g*.
10. Carefully pour off the isopropanol without dislodging the RNA pellet.
11. Add 500 μL of 70 % ethanol and centrifuge for 5 more minutes.
12. Pour off the ethanol and dry the pellet by incubating the open tube for 10 min at 45 °C in a thermomixer.
13. Resuspend in 32 μL of nuclease-free water (*see* **Note 15**).
14. Use 2 μL for spectrophotometric quantitation with a NanoDrop system.
15. Bring the sample volume up to 87.5 μL with nuclease-free water and add 10 μL of 10× DNase I Reaction Buffer, 0.5 μL of RNaseOUT, and 20 U (2 μL) of RNase-free DNase I. Final reaction volume should be 100 μL.
16. Incubate mix for 30 min at 37 °C.
17. Add 200 μL of 2× T and C Lysis Solution. Vortex mix for 5 s.
18. Add 200 μL of MPC Protein Precipitation Reagent. Vortex mix for 10 s and then place on ice for 3–5 min.

19. Repeat **steps 5–13** of this protocol. Be careful not to carry portions of the white pellet when transferring the supernatant to a new tube in **step 6**. To avoid it, centrifuge for five more minutes if needed.
20. Pool together resuspended RNA from tubes 1 and 2. The final volume should be 60 μL and total RNA amount obtained should be around 30 μg.

3.4 Nascent RNA Size-Selection

1. For the isolation of RNA fragments shorter than 200 bases, follow the instructions in section 6.4 of the NucleoSpin® miRNA kit for small and large RNA species manual. *See* **Note 16**.
2. Discard the blue column (containing the large RNA fraction), and elute the small RNA fraction from the green column with 30 μL of nuclease-free water.
3. Use 2 μL to quantitate RNA. The expected yield lies in a range of 2–5 μg of RNA.
4. Bring the sample volume down to 45 μL with a SpeedVac system, or similar. *See* **Notes 17** and **18**.

3.5 Total RNA Extraction for Conventional Transcriptomic Analysis

The sample collection for total RNA extraction can be performed in parallel with the sample collection for the BioGRO.

1. Allow cells to grow to the desired OD_{600} (typically 0.5–0.6).
2. For each sample, an aliquot of 50 mL cells is needed (corresponding to 6×10^8 cells).
3. Collect cells by centrifugation in a falcon tube at $4400 \times g$ for 2.5 min.
4. Discard the supernatant and submerge the pellet-containing tube in liquid nitrogen for flash freezing.
5. Transfer the frozen pellet to −20 °C. Keep the tube in the freezer until needed.
6. Slowly thaw cells on ice and proceed with the same RNA extraction method described in Subheading 3.3.
7. Due to the higher amount of RNA extracted, compared to the BioGRO method, resuspend RNA in 200 μL of nuclease-free water.

3.6 Tiling Array Direct Hybridization of BioGRO Samples

1. Follow the instructions of the *GeneChip®Whole Transcript (WT) Sense Target Labeling Assay Manual*, starting from *Chapter 5*: *Hybridization*. Use the GeneChip® Hybridization, Wash and Stain Kit (*see* **Note 19**).
2. Perform the staining and washing of the array as described in *Chapter 6* of the manual.

3. Repeat the Fluidics Station 450 protocol sequence twice consecutively to increase the signal of the biotinylated nascent RNAs (*see* Fig. 4).
4. At the end of the second Fluidics protocol, scan the array as described in *Chapter 7: Scanning*.

3.7 Tiling Array Hybridization of Total RNA Sample

1. For the preparation of total RNA/T7-$(N)_6$ Primers/Poly-A RNA Controls, follow the instructions of the *GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual*, starting from *Chapter 4: 100 ng Total RNA Labeling Protocol*.
2. For the next steps: First-Cycle, First-Strand cDNA Synthesis, First-Cycle, Second-Strand cDNA Synthesis, First-Cycle,

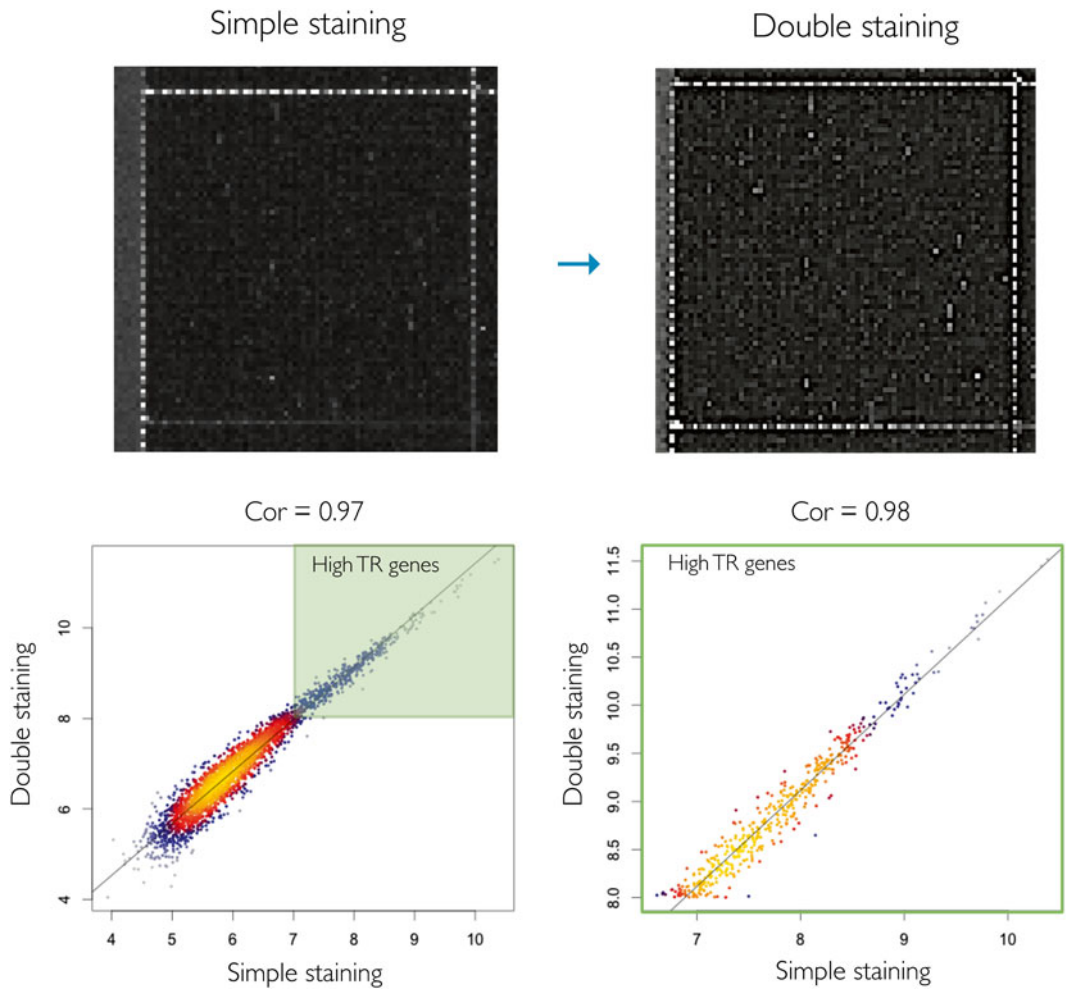

Fig. 4 Uniform re-staining of the arrays. The *top half* images show the detailed view of a tiling array quadrant region after simple and double staining. *Bottom half* graphs show how the re-staining strategy results in a uniform 2× increase in the signal

cRNA Synthesis and Cleanup, Second-Cycle, First-Strand cDNA Synthesis, Hydrolysis of cRNA and Cleanup of Single-Stranded DNA, Fragmentation of Single-Stranded DNA, and Labeling of Fragmented Single-Stranded DNA, follow instructions in Chapter 3, Procedures B–H.

3. Follow the instructions on Chapters 5 and 6 for hybridization, washing, staining and scanning of the arrays.

3.8 Adaptation of the BioGRO Protocol to C. albicans cells (See Note 20)

1. Allow cells to grow in YPD (*see* **Note 3**) at 37 °C to the desired OD_{600} (typically 0.5–0.6). *See* **Note 21**.
2. Collect cells by centrifugation in two 50 mL falcon tubes at 4400 × *g* for 3 min.
3. Slowly thaw cells on ice and add 10 mL of a 0.05 % sarkosyl solution (*see* **Note 22**). Mix by inversion.
4. Resuspend cells in 3.2 mL of 0.05 % sarkosyl and add 32 μL of 1 mg/mL DNase-free RNase A. Mix by pipetting.
5. Perform the following three washes using 0.05 % sarkosyl instead of 0.5 %.
6. Pre-warm both cells and transcription mix at 37 °C for 5 min.
7. Perform the run-on reaction by incubating the mix for 5 min at 37 °C (*see* **Note 23**) in a thermomixer, with 550 rpm agitation.
8. From this step on, follow the same RNA extraction, size-selection and hybridization procedures described for *S. cerevisiae*.

4 Notes

1. General precautions of working with RNA should be taken. Always use RNase-free water, and prepare all reagents with it. Whenever possible, work with nuclease-free materials, such as filter tips, do not touch anything that is going to be in contact with RNA without gloves, and keep your workbenches always clean. You will also minimize potential RNA degradation if you store nuclease-free materials and reagents in separate compartments inside your laboratory.
2. All homemade buffers and most solutions are autoclaved at 2 kg/cm² for 1 h to inactivate DNases and RNases.
3. Although YPD is the most common culture medium, other complete or synthetic media may also be used.
4. The cell number in each GRO experiment should be very similar between samples to avoid differences in labeling during the run-on. We estimate the real number of cells used from the amount of RNA obtained after purification. If the amount of

RNA per cell is known (this can be obtained from a series of independent RNA purifications from the known amount of cells), the number of cells is derived from it.

5. We have observed that the slow freezing of sarkosyl-treated cells causes some RNA degradation. It is recommended to freeze cell pellets immediately in liquid nitrogen or dry ice before storing them at the freezer.
6. Cell pellets can be stored more than 3 h, even for months without any negative impact on the run-on performance. We recommend storing at −80 °C for long-term periods (>1 month).
7. RNase A digestion prior to the run-on reaction is a very variable step in terms of final extracted RNA yield. We calculated that using 0.1 μg/μL RNAse A was the appropriate concentration to obtain the correct final RNA yield. However, even when using 0.1 μg/μL of RNase A, sometimes the yield is either lower or higher than expected. This variability might be due to two main things: incubation temperature, and RNase A activity. To try to optimize the first aspect we always do the incubation at 30 °C instead of at room temperature. For the second aspect, we recommend doing a trial BioGRO experiment (using unmodified UTP instead of Biotin-UTP), just before the real experiment, to test different RNase A concentrations (for example 1, 5, and 10 μg/μL) and see which one yields the expected final RNA amount (around 30 μg prior to size selection; around 2–5 μg after size selection).
8. For multiple reactions, prepare a 1/10th excess of the master mixes (transcription run-on mix and DNA removal mix).
9. Due to its photoreactive potential, it is better to avoid extended direct light exposure to Biotin-11-UTP.
10. We have checked that longer incubation times do not increase labeling. Probably, the run-on reaction is completed in only a few minutes.
11. RNA extraction was done using the "MasterPure Yeast RNA Purification Kit". This kit is designed to extract large amounts of intact RNA from yeast cells. Due to the fact that biotinylated RNAs of interest are short (50 nt on average) and present in low proportion compared to non-labeled mRNAs, some modifications to the general protocol were implemented in order to optimize the biotinylated RNA recovery.
12. Alternative RNA extraction strategies based in organic phase separation, such as acid phenol and TRIzol (Ambion), are also possible, but in our hands we recovered less biotinylated RNA than with the MasterPure kit.
13. In order to minimize loss of RNA material in the final steps of the BioGRO protocol, we used low nucleic acid retention plastic tubes (such as DNA LoBind Tubes, Eppendorf).

14. Glycogen is an inert polysaccharide that we used as carrier for the precipitation of RNA. Adding a few micrograms of glycogen significantly increased the recovery of the RNA in isopropanol precipitations. During centrifugation, it forms a visible pellet, which greatly facilitated handling of the precipitated RNA.
15. Over-drying the pellet results in a difficulty to dissolve RNA. For a complete dissolution keep the RNA pellet with water in a bench-top shaker at 45 °C for about 10–15 min. Lower temperatures and longer times may also be used. Check the dissolution by carefully inspecting while pipetting.
16. For the size selection of biotinylated RNAs, we tried many different approaches, including concentration with Amicon Ultra 0.5 centrifugal filters (Millipore), extraction from normal or low melting point agarose gels with β-Agarase, electro elution cartridges, and others, but the only one that recovered enough RNA material was the miRNA kit. This kit is designed to separately isolate both large (>200 bases) and small (<200 bases) RNA molecules from a mixed population of fragments. The 200 base-cut-off is enough to significantly enrich the run-on sample in biotinylated nascent RNAs (average size <100 bases, *see* Fig. 1).
17. We typically hybridize 5 μg of nascent RNA to the arrays. This applies for the three types of Affymetrix tiling arrays we have used (*see* Subheading 2.4).
18. *C. albicans* is a human commensal organism, with opportunistic pathogenic behavior, so general safety precautions should be taken when handling *C. albicans* cells.
19. *C. albicans*, as a human commensal organism, has an optimal growth temperature around 37 °C.
20. In our hands, *C. albicans* cells are more fragile than *S. cerevisiae* to 0.5 % sarkosyl treatments. When exposed to 0.5 % sarkosyl solutions, *C. albicans* cells break and are more difficult to recover by centrifugation, resulting in a far lower number of cells available for the run-on reaction. To avoid that, we use 0.05 % sarkosyl for initial permeabilization and post-RNase A digestion washes. It has been reported that 0.05 % sarkosyl is enough to stop ongoing transcription in the cells and prevent PIC RNA polymerases from starting new rounds of transcription [16].
21. Performing the run-on at higher temperatures (37 °C for example) resulted in better biotin-UTP incorporations. In any case we used 30 °C for *S. cerevisiae* and 37 °C for *C. albicans* in order to keep during run-on the usual culture conditions for each yeast species.
22. If you want to check/optimize for the biotinylation efficiency and RNA trimming parameters, you can run an electrophore-

sis gel (native, 2 % agarose) and then transfer the RNA to a nylon membrane using a standard Northern protocol. Once transferred, crosslink the RNA to the membrane with 50 mJ of UV radiation with a GS Gene Linker (Bio-Rad), or similar, and detect it by streptavidin-horseradish peroxidase (Streptavidin-HRP, Pierce).

23. Purity of your biotinylated RNA (proportion of labeled vs. non-labeled) can be estimated by means of a dot-blot hybridization assay, comparing your final extracted RNA against a synthetic, biotinylated RNA. Briefly: equal starting amounts of each RNA (we start with 20 ng) are placed on a nylon membrane as 1 μL dots, followed by a number of serial ½ dilutions (typically 5–8). Once dots are deposited, the membrane is air-dried for 20 min, cross-linked, and biotin signal detected as in **Note 17**.

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