Nascent RNA sequencing of unperturbed newly divided cells

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Abstract

Establishing a definitive cell cycle progression has been one of the fundamental aims of cellular biology. Its importance lies in gaining insight into the basic processes of life as well as the functions of mutant cell cycle pathways in promoting cancer by replication deficiencies and loss of checkpoint control. Currently used methods to control cell cycle and synchronize cells, function by halting cell cycle progression. Such harsh methods are detrimental to the cell and insufficient to provide an accurate reflection of the cell cycle. This study focused on replicating and confirming the efficiency of a technique developed by Helmstetter, called the “Baby Machine,” that can produce new born cells with little to no perturbations. Using this in conjunction with a short pulse RNA labelling technique, called Bru-seq, allowed the capture and RNA sequencing of synchronized cells and its nascent RNA. Here we show the first glimpse into the transcriptional profile of newly divided cells as well as novel rapid exon splicing and transcription read-through processes.
Sequencing of actively produced RNA in newly divided cells with little to no stress

Popular Science Summary

Luke Parks

The cell cycle is one of the basic mechanisms of life, during which occurs cell growth and development. Of particular significance is the progression of normal human cells versus mutated cancer cells, as cancer often stems from disruptions in the cell cycle to allow uninhibited growth. RNA sequencing techniques can allow an in-depth and comprehensive view of what the cell is doing at any given time. The cell uses RNA in a multitude of functions, including to make proteins, and its production is very sensitive to any stresses or external factors. Previous research in this area has used high stress methods to bring all the cells in a population to a desired point within the cell cycle and halt them there until released. By causing such significant disruptions to the cell’s physiology, the enormously sensitive mRNA production could be changed drastically. Therefore, results of these methods cannot be taken at face value. In this paper, we use a method that has been shown to synchronize cells in a low stress environment to collect populations of newly divided cells to perform a time specific RNA sequencing technique known as Bru-seq.
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Abbreviations

BrU bromouridine
BrUTP bromouridine triphosphate
BrdU bromodeoxyuridine
BSC biosafety cabinet
CSM cell synchronizing machine
ConA concanavalin A
DAVID database for annotation, visualization and integrated discovery
DMEM Dulbecco's Modified Eagle's medium
DNA deoxyribonucleic acid
EtOH ethanol
FBS fetal bovine serum
GSEA gene set enrichment analysis
IMDM Iscove's Modified Dulbecco's media
L-15 Leibovitz's L-15 medium
NES normalized enrichment score
PBS phosphate buffered saline
P-D-L poly-D-lysine
PI propidium iodide
RNA ribonucleic acid
RPKM reads per kilobase per million reads
RPMI 1640 Roswell Park Memorial Institute medium 1640
1 Introduction

One of the core processes of biology and thereby life, is the growth and division of cells. Through the cell cycle, an intricate arrangement of checkpoints and control systems are in place to prevent impaired cells from replicating. Its vital role in halting the early stages of cancer is perhaps the most well-known, as the loss of regulation leads to uninhibited cellular division (Kastan & Bartek 2004). To this end, synchronizing a population of cells was the first step to interrogating the complex mechanisms of the cell cycle. There are many methods that allow for the synchronizing of cell cycle phases, whether it be based on the use of temperature, starvation, or through a variety of drugs (Rosner et al. 2013). Often it relies on halting the progression of cells throughout these phases by the inhibition of vital cell cycle proteins. As the cells all reach the prescribed phase, they are then signaled to continue normal growth, this time in a synchronized fashion. While these methods are appropriate in certain cases, they are perturbing to the cells and lead to erroneous conclusions. Despite this, there have been a number of otherwise well conducted attempts to characterize cell cycle transcription profiles using such methods (Liu et al. 2017). Processes associated with the cell cycle itself may be inadvertently affected more than intended as forcefully halting cell cycle prevents normal division and is detrimental to the cell (Pardee & Keyomarsi 1992). With the sensitivity of ribonucleic acid (RNA) transcription to external effectors, cell cycle synchronization through chemical perturbation may not provide an accurate reflection of cell cycle transcription.

A relatively unknown and unused technique developed by Helmstetter and Cooper, called the “Baby Machine,” provides what they refer to as a “minimally disturbed, artifact-free, well-synchronized, mammalian cells” (Cooper 2002). The basis of this method involves the binding of cells to a membrane (Figure 1, Step 2). Once bound, the apparatus with the membrane inside, is flipped and media is run through at a steady rate. This media flows across the cells providing media consistently. As a cell divides the newborn unattached cell is washed off with the media into a collection tube.

Figure 1. Helmstetter/Cooper “baby machine” layout (Thornton et al. 2002). © 2009 BioTechniques. Used by Permission.
Helmstetter and Cooper focused their research with the machine primarily on establishing the technique and demonstrating its potential. By measuring deoxyribonucleic acid (DNA) content and cell size they were able to show that the cells that they collect maintain synchronization far better and longer than any other methods tested (Thornton et al. 2002). Further experiments in the expression of cell cycle dependent proteins revealed that the current model of determining cell phase by protein expression is skewed due to cell cycle perturbation in previous studies (Cooper et al. 2007). Through this it can be shown that the use of perturbing agents for cell cycle experiments creates a confound that prevents the results of such experiments from being reliable.

In addition, most studies use standard sequencing, RNA-Seq, which assesses the steady-state levels of RNAs of the cells, as opposed to nascent RNA, which reflects ongoing transcription more accurately. Sequencing of nascent RNA can be accomplished by pulse labelling with bromouridine (BrU) using a technique called Bru-seq (Paulsen et al. 2013). Utilizing this method, RNA that is transcribed during these pulse labelling periods may be harvested and sequenced separately from total RNA. BrU, an analog of uridine, becomes triphosphorylated into bromouridine triphosphate (BrUTP) in cells. Once in this form, it is nonspecifically incorporated into newly transcribed RNA at a similar rate as uridine. A higher concentration of BrUTP stochastically produces RNA with a higher percentage of BrUTP. RNA containing BrUTP is selectively captured by anti- bromodeoxyuridine (BrdU) beads for sequencing. These time pulse labels can help to determine direct effects of a stimuli or condition on active transcription without it being masked by pre-existing total RNA.

This project aims to bring clarity to transcription regulation during the cell cycle and more importantly a stepping stone from which others may perform new studies. By developing a protocol that provides a way in which to sequence RNA transcribed in short time periods during the cell cycle, numerous advances in cell cycle studies can be made. It will open a novel area of research previously unavailable. From these studies, medically related developments could potentially be made.

2 Materials and Methods

2.1 Cell Culture

All cells grown for the study were suspension cells. Human K562 leukemic cells were grown in Iscove's Modified Dulbecco's media (IMDM) or Roswell Park Memorial Institute medium 1640 (RPMI 1640) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 5% (vol/vol) CO₂. Mouse L1210 lymphocytic leukemic cells cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS and 1% penicillin-streptomycin in 5% (vol/vol) CO₂ or in Leibovitz's L-15 medium (L-15) with 10% FBS and 2g/L dextrose in CO₂ independent incubator. Human U937 histiocytic lymphoma cells grown in RPMI 1640 with 10% FBS and
1% penicillin-streptomycin in 5% (vol/vol) CO₂. Human GM12878 B-lymphoblastoid cells grown in RPMI 1640 with 15% FBS and 1% penicillin-streptomycin in 5% (vol/vol) CO₂.

2.2 Cell Synchronization

Here we define the protocol of the “Baby Machine” cell synchronizer, which was developed following Helmstetter’s approach (Helmstetter 2015).

2.2.1 Protocol

All materials to be used for the protocol were autoclaved and kept sterile. The machine was assembled inside a biosafety cabinet (BSC) as follows: a metal ring, rubber washer, ceramic funnel, rubber washer, mesh screen, 142 mm diameter nitrocellulose membrane with 0.22 um pores, rubber washer, polyacrylamide ring, rubber washer, and the other metal ring. Once the machine was assembled it was placed in the 37°C incubator overnight, along with tubing and glass beakers. Cell culture media and 148 mL of phosphate buffered saline (PBS) was heated in the water bath until 37°C.

The machine was attached upside down, with the membrane facing upward, to a vacuum flask. From a 500 µg/mL stock, 2 mL of cell adhesive poly-D-lysine (P-D-L) was added to 48 mL of the 37°C PBS to make a 20 µg/mL solution of P-D-L. It was poured gently onto the membrane and vacuumed through at a rate of approximately 1 mL/s. The remaining 100 mL of 37°C PBS was poured and vacuumed at 2-3 mL/s. Exponentially growing cells kept at >300,000 cells/mL and totaling to approximately 7.5*10^7 cells were then gently poured over the membrane and vacuumed at 1 mL/s. When approximately 20 mL of media was left, it was poured off to prevent drying the membrane out with the vacuum.

The machine was then flipped so that the cell phase of the membrane is facing down. The machine was leveled and positioned over a beaker. Media was used to fill the funnel to the top. A tube connecting a pump and media reservoir was attached to the funnel and the pump was set to 10 mL/min for 3-5 minutes. Following this the flow rate was lowered to 2-3 mL/min and kept consistent throughout the remainder of the experiment. The media must flow across the surface of the membrane evenly and collect at the center in a single droplet. These droplets were caught in an appropriately sized culture flask. The cells bound to the membrane grow and when they divide one of the newly divided cells will wash off with the media and collected as “newborn” cells. A depiction of the assembled machine can be seen in Figure 1. Each experiment was prefixed by cell synchronizing machine (CSM) and numbered by experiment order.

2.2.2 Cell Binding Affinity

Proficient cell attachment is necessary for the isolation of newborn cells to work. To determine proper binding efficiency of the adhesive, a count of the cells in the 20 mL of pour off and the first 30 minutes of run was made. Neither the 20 mL pour off, or the 30 minutes of elution should be less than 6% of the original total number of cells. Together they should sum
as 18-25% of the original number. Higher than this indicates a weak binding affinity and the possibility for asynchronous cells to fall off. Lower percentage suggest too strong adhesion and that even newly divided cells would not detach.

2.2.3 Age Distribution and Elution

A well performing experiment should also be reflected by the rate of cells collected per minute. In an asynchronous exponential culture, there are found a defined distribution in the age of cells (Figure 2) (Krueger & Wilson 2011). This distribution is determined by the M’Kendrick-von Foerster equation (Equation 1) (M’Kendrick 1925). It is theorized that in an exponentially growing population the ever-dividing cells creates a larger number of early G1 phase cells with steadily decreasing amounts, thereby S and G2 having fewer than the last. In addition, cell size correlates with the age of the cell. As the cell goes through the cell cycle it grows to accommodate the needs of the cell. Replication of DNA occurs in S phase and then the division of the cell in G2. For this reason, cell size measurements also provided a useful indication of the age and purity of the cells in a sample.

Figure 2. A schematic representation of the age distribution of exponentially growing cells through the cell cycle. The distribution depicts that the probability of finding a cell at zero age (the beginning of the cycle) is twice that of finding a cell at age Te (the end of the cycle). (Krueger & Wilson 2011). Cancer Cell Culture, Flow Cytometric DNA Analysis of Human Cancers and Cell Lines, 731, 2011, 361, Sarah A. Krueger, George D. Wilson, 2011 “With permission of Springer.”

\[ \frac{\partial n}{\partial t} + \frac{\partial n}{\partial a} = -m(a)n \]

Equation 1, M’Kendrick-von Foerster equation (M’Kendrick 1925). Variables: population density \( n \), age \( a \), time \( t \), death function \( m(a) \).

Applying this model to the elution of cells from the “baby machine”, results in an elution curve that reflects the age distribution of the asynchronously dividing cells. This means that because the cells that are attached to the membrane are from an exponentially growing asynchronous population, the later phase cells which are fewer in number will be the first to
divide. As time goes on and the initial G1 population reaches G2, there will be a far larger population of cells that will divide and elute. This can be seen in Figure 3 from Helmstetter’s paper (Thornton et al. 2002). The top lines are the theoretical elution rate of cells/min, while the bottom dots are experimental results.

![Figure 3. Helmstetter growth curve. Cells eluted increase in number over time reflecting the M’Kendrick-von Foerster Equation (Thornton et al. 2002). © 2009 Biotechniques. Used by Permission.](image)

Counts for this project were done by a Countess II Automated cell counter. Elution was collected and pipetted onto slides for cell counts as well as cell size measurements.

### 2.3 Flow Cytometry

Staining for flow cytometry provided the objective determination of cell cycle phase distribution. This was done by using propidium iodide (PI) to stain the DNA of cells. Cells are spun down and media vacuumed. 0.5 mL of PBS is used to resuspend cells. 0.5 mL of cold 100% ethanol (EtOH) is added dropwise to cells while vortexing. They are then incubated at -20°C for a minimum of 40 minutes. Cells are then spun down again and EtOH decanted gently. 0.5 mL of a solution of PI/RNase A with 50 µg/mL PI and 100 µg/mL RNAse A in PBS was added and vortexed. Cells were incubated in the dark for at least 40 minutes before flow cytometry. PI works by intercalating nucleic acids and fluoresces with an emission of 617 nm when exposed to a 488 nm laser. Flow cytometry detects this fluorescence at a single cell level and uses the intensity as a measurement to quantify the amount of PI bound DNA in a cell. A human cell is diploid and in G1 will have two sets of chromosomes making it 2n. Once the cell enters S phase, it begins the process of DNA replication to duplicate its genome. As the DNA content increases there will be a shift from 2n towards 4n. Once reaching 4n the DNA has doubled and the cell is considered to be in G2. Therefore, relative PI intensity
indicates where in the cell cycle a cell is as well as the overall distribution of a population of cells.

Samples were collected from the “baby machine” for durations anywhere from 6-20 minutes. These were either fixed immediately or aged through various points in the cell cycle and then fixed. To fix, cells were pelleted and media removed. Cells were resuspended in 0.5 mL of PBS, and then 0.5 mL of ice cold EtOH was added dropwise while vortexing. Samples were incubated at -20°C for at least 30 minutes. Cells were again pelleted and EtOH was poured out and then tubes were blotted. A PI/RNAse A solution was prepared by adding 50 µg/mL of PI and 100 µg/mL of RNAse A to PBS. The RNAse is used to remove RNA and prevent PI binding. Samples were resuspended by adding 0.5 mL of PI/RNAse A and then vortexed for 5 seconds to minimize cell doublets.

After incubating in the dark for 35 minutes at room temperature, samples were loaded into a flow cytometer for analysis. For proper analysis 10,000-15,000 cells was set as the upper capture limit. An asynchronous exponentially growing population was used to set gates for G1 and G2. Once finished, files were processed with ModFit LT™ to calculate percentages of G1, S, and G2 in the population. Forward scatter height vs area was used to eliminate cells clumped together and formed doublets. When two new-born cells are attached, they give off a similar emission to a cell in G2 as there will be double the DNA content. However, together doublets are larger than a cell in G2 and can therefore be eliminated based on size. %CV is the coefficient of variation from G0/G1, and helps determine the goodness of fit. Figure 4 is an example of a typical flow cytometry result of an asynchronous exponentially growing population.

![Flow Cytometry Graph](Image)

Figure 4. Example flow cytometry and ModFit LT™ analysis of asynchronous exponentially growing human U937 cells.
2.4 Bru-seq

Once a well-functioning cell synchronizing experiment was established, BrU labeling was performed following the outline of Figure 5 in experiment CSM18. 20-minute samples from the elution of the “baby machine” were collected in T75 cell culture flasks. Immediately following, a 37°C solution of BrU was gently added to a final concentration of 2 mM. BrU labeling of nascent RNA was performed for 30 minutes, after which samples were transferred to a centrifuge tube and spun down at 4°C for 4 minutes. Media was carefully removed from the tube. Trizol was added to samples and pipetted to lyse cells and suspend RNA. A total of 5.6 million cells were collected and pooled in this manner. The sample was stored at -80°C until sequencing. A total RNA isolation was performed with a chloroform/phenol extraction followed by an isopropanol precipitation, then washed with EtOH and resuspended in DEPC-H2O. BrU-labelled RNA was separated using anti-BrdU magnetic beads and stored at -80°C. Library preparation used an Illumina Kit to convert RNA to cDNA with random primers by PCR. Second strand cDNA was made from the first cDNA strand with RNA digestion. The double stranded cDNA had ends repaired and poly-adenylated to ligate adaptors. The sample was size selected to 300 bp by agarose gel and purified. cDNA was amplified and cleaned. Sample was sent for sequencing to the University of Michigan sequencing core.

Figure 5. Overview of new-born cell capture and BrU labeling and sequencing performed in CSM18. Newly divided cells washed off the membrane and were collected in 20 min fractions. mRNA was labeled with 2 mM BrU for 30 min, cells were lysed in Trizol and pooled. Total RNA was isolated and BrU-labeled RNA captured by anti-BrdU beads. Reverse transcription into cDNA was performed, and these were used to synthesize the second strand of cDNA. Samples were sent for Illumina deep sequencing.
Total RNA isolation, BrU-RNA capture, and Illumina library prep was performed by Michelle Paulsen, the lab manager of the lab who is highly experienced in these procedures.

### 2.5 Gene Ontology

Gene set enrichment analysis (GSEA) and database for annotation, visualization and integrated discovery (DAVID) gene ontology were performed to determine enriched pathways. All genes \( \geq 300 \) bp detected with reads per kilobase per million reads (RPKM) \( \geq 0.5 \) in the sample were used as the full gene list. For GSEA the full gene list along with fold changes compared to an asynchronous sample was analysed. Based on the fold change and the pathways different genes are previously reported to play a role in, GSEA will calculate a normalized enrichment score (NES) for pathways it determines are significantly positively or negatively enriched. DAVID on the other hand uses the full gene list as a background and then has genes that were \( \geq 2 \)-fold upregulated or downregulated compared to an asynchronous sample imported without fold changes. By analysing upregulated or downregulated genes against the background, it calculates a \(-\log_{10} p\)-value for gene pathways and displays those found to be significant.

### 3 Results

#### 3.1 “Baby Machine” Establishment

##### 3.1.1 Old Machine

A number of experiments were required to achieve satisfactorily synchronized cells from the “baby machine.” Here was performed several variations of the reported Helmstetter protocol to collect a high fraction of newly divided cells. These involved use of different binding agents, incubation times, machines, flow rates, membranes, and cell types (Table A1). Figure 6 demonstrates the cell cycle analysis results measured by flow cytometry. At CSM11 with human U937 cells and concanavalin A (ConA) membrane adhesive there was seen an increase in G1 phase percentage to 86.44, as calculated by ModFit LT™. Following experiments with mouse L1210 and human K562 with P-D-L as the membrane adhesive retained the increase in G1 percentage. CSM19 was an experiment attempted with human non-cancerous GM12878 cells. Their tendency to clump resulted in cells bunching together on the surface of the membrane and falling off together, contaminating the collection of new-born cells. L1210 cells produced the highest purity, due to the faster division time of 9 hours compared to U937 and K562 with approximately 24-hour division times. With the potential for almost three times the number of cells dividing at any given time, L1210 newly divided cells dilute the already low portion of asynchronous cells collected.
Figure 6. Average cell phase percentages of experiments. First four samples are asynchronous averages with each cell type. CSM1-CSM20 represent experiment number with the cell type used attached.

Figure 7. Flow cytometry of CSM5 control cells (a) and captured new-born sample (b) stained with PI.

An example of a flow cytometric analysis of a non-functional experiment is exhibited in Figure 7, where CSM5 with K562 cells retained a large percentage of S phase cells. While there is a noticeable increase in G1, it was not substantial. Cell membrane affinity tests were performed to quantify the percentage of cells that would become bound to the membrane to determine if adhesion was satisfactory. Table 1 shows a positive cell affinity test that produced binding of 79.71% of the original number of cells. Adhesion of cells was
conditional depending on media type, cell type, and CO2 percentage. The best adhesion was found with P-D-L at 20 µg/mL at 5% CO2.

Table 1. Cell membrane affinity measurement of K562 cells with 20 µg/mL P-D-L from CSM17.

<table>
<thead>
<tr>
<th></th>
<th>Total Cells Counted</th>
<th>Total Cells Percentage (%)</th>
<th>Average Cell Size (um)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting Amount</td>
<td>55530000</td>
<td>100.00</td>
<td>20.63</td>
</tr>
<tr>
<td>Pour Off</td>
<td>8375000</td>
<td>15.08</td>
<td>20.13</td>
</tr>
<tr>
<td>Elute (30 min)</td>
<td>2890800</td>
<td>5.21</td>
<td>15.86</td>
</tr>
<tr>
<td>Pour+Elute</td>
<td>11265800</td>
<td>20.29</td>
<td>17.99</td>
</tr>
<tr>
<td>Bound</td>
<td>44264200</td>
<td>79.71</td>
<td></td>
</tr>
</tbody>
</table>

Figure 8. Cell elutioin counts with mouse L1210 cells throughout a full cell cycle from CSM16. Trendline calculated by Microsoft Excel.

As discussed in Materials and Methods with Equation 1, due to a greater number of cells being recently divided and decreasing in number till the end of G2, an elution curve consistent with Equation 1 is found in a cell synchronizing experiment. Figure 8 of CSM16 with mouse L1210 cells illustrates the elution curve expected based on the equation and previous findings by Helmstetter in Figure 3. As the duration of the cell cycle progresses, the dividing cells will be those that were originally in G1 at the start of the experiment. Therefore, more cells will be eluted per minute. Because the curve shape is consistent with our expectations, a large portion of eluted cells must be newly born. Discrepancies in the curve compared to Figure 3 is a consequence of the sample number of cells. The Countess Cell Counter II can count a large number of cells, but not the thousands of cells a Coulter Counter is capable of, as used by
Helmstetter (Thornton et al. 2002). A flow cytometric analysis of the cells captured in the experiment revealed a collection of 88.98% of cells being in G1 (Figure 9). As the experiments were producing high fractions of newly divided cells, an experiment was performed to BrU label and sequence the cells.

![Flow cytometry graphs](image)

Figure 9. Flow cytometry of CSM16 control cells (a) and captured new-born sample (b) stained with PI.

### 3.2 Bru-Seq of Newly Divided Cells

Once the use of the “baby machine” was optimized and confirmed as reproducible an experiment was set up to collect new-born cells and incubate with BrU for labelling and capture of nascent RNA as described in Materials and Methods. Human K562 cells were used for the sequencing of newly divided cells. These cells were collected in 20-minute fractions and then labelled with BrU for 30 minutes before lysing with Trizol. Cells ranged in age at the beginning of labelling from 0-20 minutes in age and at the end 30-50 minutes in age. This categorizes cells as early G1, with a 24 hour division time and previous reports suggesting a 6 hour G1, 8 hour S phase, and 6 hour G2 (Hoyes et al. 1992).

#### 3.2.1 Membrane Affinity and Flow Cytometry

Cells were bound to a nitrocellulose membrane using 20 µg/mL of P-D-L. To confirm appropriate binding affinity, the cell number from the pour-off and first 30 minutes of elution were counted (Table 2).

Of the initial 79 million cells, approximately 62 million cells remained bound to the membrane. This represents a portion of 78.01% still bound to the membrane, confirming cells bound with the proper affinity.
Samples for flow cytometry were taken at the beginning as well as during the experiment, and immediately fixed in EtOH. These were stained with PI as described in methods and analysed by flow cytometry for cell cycle phase determination. More than 10 thousand cells were analysed by flow cytometry for each sample. The exponential asynchronous sample in Figure 10a displays a standard pattern for cell cycle. Both Figure 10b and c contain greater than 80\% of cells in G1. This is more than a 40\% increase from the control. This change suggests that a large portion of the cells collected were new-born cells.

Figure 10. Flow cytometry with PI stain of CSM18 control cells (a) and captured new-born samples (b-c). New-born samples collected at beginning and middle of experiment.
3.2.2 Bru-seq Upregulation and Downregulation

Sequencing results reported good capture of BrU labelled RNA (Table 3). As ribosomal RNA (rRNA) makes up 80% of total RNA in a cell, it can provide a basis of a background. This background can be defined as non-BrU labelled RNA picked up non-specifically. rRNA is consistently produced in the cell at a small percentage of total RNA, but unlike most RNA is accumulated to higher concentrations. The new-born cell collection sample, K562CSM180h7, had an rRNA content of 10% implying low background RNA collection since it saw a reduction from 80% to 10% rRNA. In addition, percentage of intron sense versus exon sense RNA can be used to detect background and age of sample. As mRNA ages, it will become spliced and intron RNA degraded. As seen in a sample aged 6 hours, K5626h2, compared to K562CSM180h7, there is a much higher percentage of exon sense RNA as well as a higher fractionSpliced. These suggest that K562CSM180h7 was well labelled and sequenced and should give reliable results.

Table 3. Sequencing results of new-born sample (K562CSM180h7) vs historical asynchronous exponentially growing sample (K5620h6) and historical asynchronous sample aged 6 hours.

<table>
<thead>
<tr>
<th>sample</th>
<th>rRNAReadCount</th>
<th>intron_sense</th>
<th>exon_sense</th>
<th>fractionSpliced</th>
</tr>
</thead>
<tbody>
<tr>
<td>K5620h6</td>
<td>6%</td>
<td>51%</td>
<td>9%</td>
<td>0.01109</td>
</tr>
<tr>
<td>K562CSM180h7</td>
<td>10%</td>
<td>51%</td>
<td>11%</td>
<td>0.02545</td>
</tr>
<tr>
<td>K5626h2</td>
<td>14%</td>
<td>17%</td>
<td>34%</td>
<td>0.07934</td>
</tr>
</tbody>
</table>

From this sample 8,615 genes greater than 300 bp were sequenced with expression levels above 0.5 RPKM. RPKM is defined as reads per kilobase per million reads which is a way making expression relative to baseline. Of these, 873 were upregulated by 2-fold or greater compared to historical asynchronous exponentially growing sample K5620h6. Genes that were downregulated by 2-fold or more totalled to 835 genes. Instances of both are demonstrated below. Lines found above the x-axis correspond to genes on the forward DNA strand, and these are displayed in green above. Lines below the x-axis are genes that are found on the reverse strand and are shown in red above. Figure 11 illustrates nine genes which are upregulated in newly divided cells. The blue line represents RPKM of the new-born sample, K562CSM180h7, and the red line represents RPKM of the asynchronous sample, K5620h6. Several correlate with previous studies, though some represent new findings. NES’s translated protein, nestin, is known to disassemble intermediate filaments during mitosis, and its continued transcription in this sample could mean this process goes into G1 (Chou et al. 2003). Mitotic spindle formation and cell cycle progression are both consequences of CBX2 (chromobox 2) (Clermont et al. 2016). Of particular importance to cells is RBM19 (RNA binding motif protein 19), which is involved with regulating ribosomal biosynthesis (Zhang et al. 2008). A region of chr19 saw an upregulation as well, suggesting clusters of coordinated upregulation for early G1.
Figure 11. Examples of upregulated genes in new-born vs asynchronous cells. Bru-seq traces for: MAP1A, CBX2, NES, UBTF, NES, CSK, ZNF74, Chr19 Cluster, RBM19, MYC. Newly born (blue) and asynchronous (red) cells. Nascent RNA sequencing reads expressed as RPKM (reads per kb per million reads). Traces above baseline are forward strand genes (green), while traces below baseline are reverse strand genes (red).

Figure 12. Examples of downregulated genes in new-born vs asynchronous cells. Bru-seq traces for: TP53, PMAIP1, TRIB3, BHLHE40, CD69, EGR1, ANXA1, SLC2A3, CCNG1. Newly born (blue) and asynchronous (red) cells. Nascent RNA sequencing reads expressed as RPKM (reads per kb per million reads). Traces above baseline are forward strand genes (green), while traces below baseline are reverse strand genes (red).
Of the downregulated genes, TP53 is one of the most important (Figure 12). This gene encodes p53 which is shown to enforce apoptosis or cell cycle arrest when upregulated in mitosis and early G1 (Yang et al. 2017). A downregulation of the gene would mean the cells are growing unperturbed on the membrane and when eluted are proliferative. PMAIP (NOXA) is a p53 associated gene and contributes to its upregulation for apoptosis by binding with MCL1 which releases BCL2L11 (Zhao et al. 2014). EGR1, encodes early growth response 1, a transcription factor that has been correlated with cell cycle progression, but there have been conflicting reports as to its method of action. Studies have found its roles in mitosis, early cell growth, as well as p53 signalling (Li et al. 2013, Liao et al. 2004). It is likely that these functions all come into play during the cell cycle. However, these results indicate that early G1 sees a downregulation.

3.2.3 Gene Ontology
Both GSEA and DAVID gene ontology programs were used to analyse enriched pathways from this data set. This was done by comparing ≥2-fold upregulated or downregulated genes against the background of total genes. Upregulated GSEA saw a high NES for Myc targets (Figure 13). Myc is very often related to cell cycle progression (Bretones et al. 2015). An upregulation of targets would trigger a cascade of signals to prepare the cell for continuation through the cell cycle. Pyrimidine and purine’s metabolism function is to restore the much-needed nucleic acids to prepare the cell for the replication of DNA to be performed in S phase. DAVID shows that transcription is generally upregulated. Meaning transcription factors and gene transcription are things that would see an increase to account for the change in needed genes for newly divided cells as well as shifting from one phase to another. Rap1 signalling promotes several downstream effects including cell-cell junctions (Kooistra et al. 2007). Notch signalling is upregulated in GSEA and DAVID. This is a transmembrane protein that also has numerous downstream changes depending of the effector, many of which relate to cell differentiation (Hori et al. 2013). Both GSEA and DAVID saw large upregulation of taste transduction. The reason for this upregulation is unclear, but could lead to the discoveries of cell cycle specific genes that were previously thought to be unrelated.

Downregulated pathways determined by GSEA and DAVID shared many pathways. Many of the downregulated pathways were apoptosis related, including; apoptosis, p53 pathway, and TNFα signalling. As cells exit mitosis, they can be committed to proliferation or quiescence. By inhibiting factors related to apoptosis and p53, the cells are primed for proliferation. The downregulation of KRAS signalling could indicate that its role in promoting cell growth factors occurs later in the cell cycle (Sunaga et al. 2011). The ribosome was another ontology class that was found to be downregulated in both GSEA and DAVID.
3.2.4 Exon Splicing and Transcription Read-Through

Splicing is an important part of the life cycle of RNA and occurs co-transcriptionally. A 30-minute labelling period does not show evidence of more than 20-30% splicing in a large portion of its RNA, but if the time is extended a greater portion of RNA obtained is spliced. Our lab has preliminary evidence that splicing overall is not very efficient leading to a large number of aberrantly spliced transcripts that are removed over the next two hours by a nuclear RNA quality control mechanism executed by the nuclear RNA exosome. Hence why in Table 3, the aged sample, K526h2, has a much higher exon_sense_total percentage as well as fractionSpliced. The new-born sample contained an appropriate amount of these. However, a few specific genes saw a higher degree of co-transcriptional splicing than in the asynchronous samples. Of these, NEFH and PARP1 were the most noticeable. These genes code for proteins that make up the heavy chain of neurofilaments and poly (ADP-ribose) polymerase 1 respectively (Dong et al. 1993, Wang et al. 1995). Thus, these results suggest that the rate of splicing for some specific genes may be regulated in a cell-cycle dependent manner.

As can be seen in the top two graphs of Figure 14, there is significant increases in RPKM of the new-born sample (blue) at the exons, represented by the shaded region of the gene. This can be compared to an asynchronous sample aged 6 hours (green), which follows a trend of heightened expression at the exons. Due to the specificity of splicing as well as these genes
becoming highly upregulated, it can be theorized that there are mechanisms that allow these genes to bypass the ordinarily imposed splicing time.

![Figure 14. Novel rapid exon splicing and transcription read-through in selected genes in newly born cells. Exon splicing (top) in NEFH and PARP1 within a 30 minute BrU labeling period. Newly born (blue), asynchronous (red), asynchronous 6-hour, K5626h2 for NEFH and K5626h5 for PARP1, (green) cells. Transcription read-through of mRNA in SSBP1 and NPM1. Traces above baseline are forward strand genes (green), while traces below baseline are reverse strand genes (red).](image14)

Additionally, there were also discovered a few instances of transcriptional read-through (Figure 14 bottom). Transcriptional read-through is when an mRNA transcript extends past the defined limits of the gene. The examples shown are for SSBP1 and NMP1, also known in its protein form as single stranded DNA binding protein 1 and nucleophosmin respectively. For SSBP1 the expression was elevated in the read-through despite similar levels of gene transcription. Its read-through continued into TAS2R3-5. NPM1 saw a similar read-through into FGF18. Thus, transcriptional termination may be regulated in a cell-cycle-dependent manner for some genes to allow transcriptional read-through to upregulate downstream genes.

### 3.2.5 Transcription Dynamics

In previous unpublished work it was found that after labelling nascent RNA for 20 minutes with BrUTP and staining with florescent anti-BrdU antibodies, approximately 180 foci appears per cell (Figure 15). Florescence appears at sites of nascent RNA, but rather than being evenly distributed throughout the nucleus they are localized. This correlates with work describing transcription factories, in which a large number of RNA polymerase II aggregate to transcribe RNA (Papantonis & Cook 2013). Despite the low number of transcription factories, 8,615 genes were still detected in the new-born sample, comparable to asynchronous K562 cells which produce roughly 8,000 to 9,000 genes.
4 Discussion

This study sought to establish the protocol of the “baby machine” for the sequencing of nascent RNA in newly divided cells. While there have been many studies of the cell cycle over the years, the dominating techniques utilize cell cycle perturbing methods. The inherent responsiveness of the cell to external effectors makes these studies unreliable. RNA expression is particularly responsive, and harsh reagents or growth conditions mediate instantaneous shifts in the transcriptional landscape that are retained for many cell cycles. Therefore, this experiment relied on the “baby machine” for capturing minimally unperturbed cells.

Many experiments were performed, and the protocol fine-tuned to produce populations with high percentages of cells in G1. Well-functioning experiments were determined by monitoring cell adhesion and elution throughout the cell cycle. Achieving a proper balance between high enough membrane affinity to remain bound and low enough that new-born cells can still detach, is the first step. Aiming for approximately 80% of cells bound provided a good approximation of adequate binding. Staining with PI for flow cytometry provided an unbiased detection of cell cycle phase percentages.

Elution of high percentages (>80%) of G1 phase cells was achieved in mouse L1210, human U937, and human K562 cells. Consistency of results was attained with these cell lines, with P-D-L as the primary membrane adhesive. Limitations to this method lie in the need for suspension cells with minimal clumping, as evidenced by GM12878 results. As cell cohesion
endured the washing of media across the surface of the membrane, cells remained suspended together before falling off.

Human K562 cells were captured from the “baby machine” and labelled with BrU for the collection of nascent RNA. Collected RNA came from a majority of cells that were 0-20 minutes post division at the beginning of labelling. As the division time of these cells is 24 hours, with a G1 estimation of 6 hrs, these cells can therefore be classified as being in the early stages of G1. Coming just after division, lingering effects of mitosis should remain, but more importantly a shift towards growth and preparation for replication should be seen.

Much of what is currently known about the cell cycle comes from studies involving perturbing reagents. Our data does not mimic that of previous studies, and due to the sensitivity of the cell, should not be expected to. As the first study of the transcription of early G1, novel insights into newly divided cells can be gleaned, as well as provide confirmation of prior discoveries. Deeper analysis will promote understanding of pathways in the cell and their impact on development and progression. Genes once thought to be limited to certain phases of the cell cycle, are shown to play a role in early G1. For instance, nestin and chromobox 2 are well studied protein that are thought to be limited to G2 in order to induce the production of mitotic genes, but here can be shown to remain upregulated. It may be revealed that early G1 has a greater function as a clean-up stage in the cell cycle to remove residue from mitosis.

Cyclins and cyclin dependent kinases play critical roles in cell cycle regulation and are therefore worth mentioning. However, as these proteins are primarily responsible for the transition of phases and newly divided cells are several hours from G1/S transition, it should not come as a surprise that many of the associated genes did not experience significant changes. CCND2 (Cyclin D2) which is involved with control of G1/S transition did not experience a 2-fold up or down-regulation of transcription, nor did its associated kinases, CDK6 and CDK4. CCNE1 (Cyclin E1), which also impacts the G1/S transition, and its kinase, CDK2 did not experience significant fold changes. CCNA2 (Cyclin A2) also associates with CDK2, but for the S/G2 transition, and did not experience any change. For the transition to M phase, Cyclin A utilizes cdk1, whose gene was downregulated by more than 2-fold. The final transition from M to G1 is directed by Cyclin B1 with cdk1, both of which were downregulated by more than 2-fold. Also associated with G2/M phase, but instead for cell cycle arrest and apoptosis, is CCNG1 (Cyclin G1). It was found to be more than 2-fold downregulated, and is shown in Figure 12. Further studies into later points of the cell cycle will elucidate the roles of cyclins in the promotion of transition between phases.

Gene ontology provided general information into the process of the cells. Many upregulated pathways had primary functions in cell cycle progression and preparation for later phases. Downregulated pathways displayed an anticipated inverse, with apoptosis and quiescence downregulated through p53 associated pathways. The G2M checkpoint and mitotic spindle were both downregulated as well, marking the distinct changes between G2 and early G1.
Even with the knowledge of which pathways are changed, it is not always clear as to why. Ribosomal pathways and their downregulation is an instance of this. Despite being such a vital process in the cell, it is not clear why it would experience downregulation in G1. Taste transduction and its significant upregulation might give rise to a new G1 specific pathway.

Rapid exon splicing of genes could prove to be an indicator of an important novel function in G1. Specificity towards a select few genes, such as NEFH, and low overall exon to intron ratio determines that this phenomenon is not the subject of error. The biological process for how it occurs can only be theorized at this point in time. Evidence seems to indicate the recruitment of a greater number of splicing factors specifically. Surrounding genes did not see an increase in splicing, allaying possibilities of storage localization during division. Other genes may experience similar splicing in later points of the cell cycle. This could prove to be a novel form of splicing that would open exploration into methods of regulation and preferential binding.

Aggregation of RNA polymerase II into transcription factories prompted a question of whether these factories assemble at particular sites in a prescribed order during the cell cycle or target RNA for transcription stochastically. If transcription factories appeared in a cell cycle dependent manner, there would be a specific set of fewer genes transcribed compared to an asynchronous population which would encompass all points of cell cycle dependent transcription factory localization. Historical samples of K562 transcribed approximately 8,000 to 9,000 genes. By the transcription of 8,615 genes in the new-born sample, it can be predicted that cells did not follow a predetermined pathway towards division, but rather occurred stochastically with an emphasis on transcription as needed.

Future research could bring into light the role of various genes in the cell cycle. Investigation will highlight the evolving role of genes as the cell transitions from one phase to the next. A full characterization of the cell cycle will lead to a greater understanding of the basic processes of life. Of particular interest will be whether rapid exon splicing plays a role in other stages, or is constrained to early G1.

5 Acknowledgement

Special thanks to my supervisor, Dr. Mats Ljungman, for his support and patience with the project.

For his advice and comments appreciation goes to Dr. Charles Helmstetter, as well as to Dr. Steven Cooper for the use of his “baby machine” set-up.

Thanks due for her continual patience with my frequent all-nighters in the lab to my wife, Allison.
For the encouragement of interest in the world around us, I would like to thank my family.
References


### Table A1. Experimental variables tested for establishing “baby machine” protocol.

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<td>~2 mL/min</td>
</tr>
</tbody>
</table>