Supplemental Experimental Procedures

Single Cell AMplification Procedure (SCAMP)

The tools available for the analysis of gene expression patterns of very small samples, including single cells, remain imperfect. They can be divided into three basic biochemistries. One method uses multiple rounds of *in vitro* transcription[1], mediated by a T7 promoter incorporated into the cDNA. This approach has been used with success for small laser capture samples and even single cells.[2-5] Nevertheless, the procedure is arduous, requires days to complete, the results are variable[6], and available commercial kits specify input RNA amounts far exceeding single cell quantities. A second method, designated RiboSpia, uses a displacement DNA polymerase reaction[7] to drive a single round of amplification. The RiboSpia OneDirect kit from Nugen is stated to work with RNA from even a single cell. A chief disadvantage is the very high cost. A third method uses PCR based target amplification. Perhaps surprisingly, this has been shown to be capable of providing faithful representation from even extremely small starting samples.[8]

Detailed protocol for PCR target amplification in single cells [optimized from the protocol[9] previously used by Cepko and colleagues.[10] 1 µl of RNA, or even a single cell, was added to 5 µl of RT-Lysis Buffer. RT-Lysis Buffer has 47 µl Lysis Buffer, 1 µl RNAseout (Invitrogen), 1 µl dNTP (2.5 mM each, Takara), 0.5 µl oligodT (20 ng/µl), 0.5 µl oligodT+N (5 ng/µl). Oligonucleotides used in these studies were supplied by Oligos Etc. (Wilsonville, OR) and contained the sequences 5'-TATAGAATTCGCGGCCGCTCGCGATTTTTTTTTTTTTTTTTTTTTT (Oligo dT) and 5'-

TATAGAATTCGCGGCCGCTCGCGATTTTTTTTTTTTTTTTTTTTTTTTNNNNNN (Oligo dT+N). Lysis buffer consists of 100 µl 10X Roche PCR Buffer, 5 µl NP-40, 50 µl 0.1 M DTT, 60 µl 25 mM $MgCl_2$, 785 µl H_2O. The samples were heated to 65^OC 2 min, cooled to 4^OC, and centrifuged. The random primer added in the initial reverse transcription (RT) step gives improved full-length representation of transcripts which improves results when using, for example, the Affymetrix Gene ST arrays, which carry probes across multiple exons, and are not strongly biased for the 3' ends of genes.

RT reactions were set up by adding 0.8 µl Superscript Mix, incubating 25^OC for 5 min, 37^OC for 30 min, heat-inactivated at 70^OC for 10 min, cooled to 4^OC and centrifuged. Superscript mix consists of 3 µl Superscript III (Invitrogen), 0.5 µl RNAseout (Invitrogen), 0.3 µl T4 gene 32 protein (New England Biolabs). For exonuclease reactions, 0.5 µl exonuclease (New England Biolabs) was added, incubated 37^OC for 30 min, heat-inactivated at 80^OC for 25 min, cooled to 4^OC and centrifuged. Tailing reactions were carried out by adding 3 µl TdT mix, incubated 37^OC for 20 min, heat-inactivated at 70^OC 10 min, cooled to 4^OC and centrifuged. TdT mix contains 0.15 µl of 100 mM dATP (Roche), 0.3 µl 10XPCR buffer (Roche), 1.37 µl H_2O, 0.5 µl TdT (Roche), 0.5 µl RNaseH (Ambion) and 0.18 µl of 25mM $MgCl_2$. PCR reactions were carried out by adding 90 µl PCR mix, and cycling at 95^OC for 1 min, 95^OC for 1 min, 37^OC for 5 min, 72^OC for 16 min, 93^OC for 40 sec, 67^OC for 1 min, 72^OC for 6 min + 6 sec per cycle, repeating the cycling conditions 34 times, 72^OC for 10 min, 4^OC forever. The PCR mix consists of 10µl 10X PCR buffer, 10µl dNTPs (2.5mM), 2µl Oligo dT (1µg/ul), 1µl LA-TAQ polymerase and 67µl of H_2O. PCR reagents were purchased from

Takara Bio. The resulting amplified cDNA products were purified using Qiagen PCR purification kit (Qiagen).

The following optimized fragmentation and labeling procedure produces target properly sized for Affymetrix oligonucleotide array hybridization. 5 μg of cDNA were fragmented in DNase I fragmentation mix containing two units DNase I (Roche) and 1X One-PHor buffer (100mM K-glutamate, 0.25mM Tris-acetate, 0.1mM Mg-Acetate). The cDNA was incubated at 37°C for 13 min, heat-inactivated at 95°C for 15 min, cooled to 4°C and centrifuged. The fragmented cDNAs were biotinylated by adding 1.5 μl of 1mM Bio-N6-ddATP (ENZO Life Scences) and 1 μl of TdT (Roche). The samples were incubated at 37°C for 90 min, heat-inactivated at 65°C for 15 min., cooled to 4°C and centrifuged.

To evaluate the quality of SCAMP data we performed comparisons with two commercial systems, Nugen RiboSpia OneDirect and Miltenyi μMACS SuperAmp, which is also PCR based. Amplifications were performed starting with 25 and 50 picograms (pg) of RNA standard that was made from whole newborn mice. Total RNA content per cell will depend on cell type, but is generally in the range of 5-30 pg per cell [11;12]. Miltenyi provided a technician that performed the μMACS SuperAmp PCR based procedure, according to their protocols and with their equipment, which includes special columns, while we carried out the Nugen OneDirect and the SCAMP protocols. Technical replicates were performed, in each case starting with the same newborn mouse homogenate standard RNA, but performing independent amplifications and microarray hybridizations, with Affymetrix Mouse Gene 1.0 ST arrays.

A total of sixteen test small sample amplifications and array hybridizations were carried out, including two using Miltenyi μMACS SuperAmp (one 25 pg and one 50 pg), four with Nugen OneDirect (two 25 pg and two 50 pg), and ten with SCAMP (three 10 pg, four 25 pg and three 50 pg). Pearson Correlation Coefficients (PCC) were calculated to provide a measure of reproducibility using GeneSpring GX 11.0.2. The PCC values generated were 0.917 for μMACS SuperAmp, and 0.924-0.949 for Nugen OneDirect and 0.860-0.941 for SCAMP. The 10 pg samples, only attempted with SCAMP, gave more noise, as might be expected, but all three systems gave excellent reproducibility.

M25	M25														
M50	0.92	M50													
D25	0.81	0.82	D25												
D25	0.79	0.82	0.92	D25											
D50	0.80	0.83	0.94	0.94	D50										
D50	0.81	0.84	0.94	0.93	0.95	D50									
S10	0.76	0.80	0.79	0.77	0.78	0.79	S10								
S10	0.77	0.82	0.79	0.78	0.79	0.79	0.86	S10							
S10	0.79	0.84	0.80	0.79	0.80	0.81	0.87	0.88	S10						
S25	0.75	0.81	0.79	0.78	0.80	0.80	0.87	0.88	0.89	S25					
S25	0.74	0.79	0.79	0.78	0.79	0.79	0.86	0.87	0.87	0.89	S25				
S25	0.71	0.77	0.78	0.77	0.79	0.79	0.85	0.86	0.86	0.88	0.87	S25			
S25	0.71	0.77	0.78	0.77	0.78	0.78	0.85	0.86	0.87	0.89	0.87	0.88	S25		
S50	0.71	0.78	0.78	0.76	0.78	0.78	0.87	0.87	0.88	0.90	0.89	0.89	0.90	S50	
S50	0.72	0.78	0.77	0.77	0.78	0.78	0.87	0.87	0.88	0.91	0.90	0.89	0.90	0.92	S50
S50	0.73	0.80	0.79	0.78	0.80	0.80	0.88	0.89	0.91	0.92	0.90	0.91	0.93	0.94	0.93

Pearson correlation coefficients for Miltenyi (M), D (OneDirect) and S (SCAMP) methods. Numbers next to M, D, S designate picograms of total RNA used.

We further tested the quality of the SCAMP data. One important measure of target amplification quality is sensitivity, or the ability to detect low abundance transcripts. Analysis of the array data indicated that SCAMP gave greater sensitivity than

either OneDirect or SuperAmp, with ANOVA results showing SCAMP detection of expression of significantly more genes, and with higher mean probe level intensities (Figure S1). In addition, for Affymetrix Gene ST arrays there is a quality metric designated “area under the curve”, or AUC. This is a measure of relative signal intensities for the exons of about 100 housekeeping genes, compared to introns for the same genes. A higher AUC therefore indicates more specific signal. The average AUC values for the 50 pg samples were 0.870 for SCAMP, 0.825 for OneDirect, and only 0.700 for SuperAmp. SCAMP gave clearly superior data as measured by this key quality metric. These overall results show that SCAMP is a cost effective, easily executed system for the target amplification of extremely small samples, yielding high quality data.

We therefore used the SCAMP procedure for target amplification of the five individual YFP positive juxtaglomerular renin producing cells.

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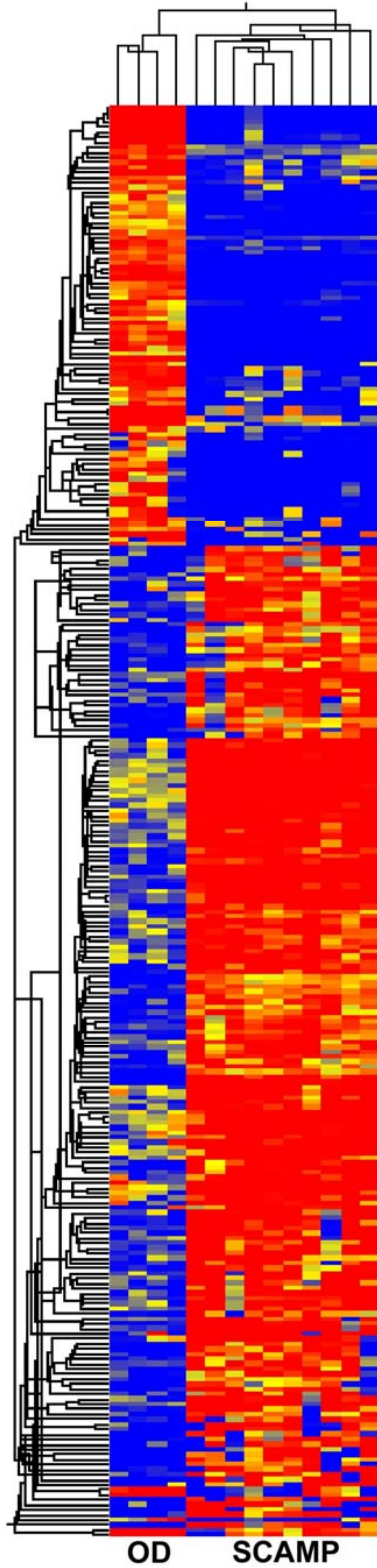

Supplemental Figure 1