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Single Cell Gene Expression Profiling: Multiplexed Expression Fluorescence in situ Hybridization (FISH)

Application to the Analysis of Cultured Cells

By: Jeffrey M Levsky, Steven A Braut and Robert H Singer

I. Introduction

Most current methods of measuring gene expression rely on averaging many cellular responses or artificial amplification steps to reach a detectable threshold of signal. In contradistinction, in situ assays circumvent these procedures to yield direct single cell expression information. Fluorescence in situ hybridization (FISH) is the gold standard for localization of nucleic acids (Fauth and Speicher, 2001; van der Ploeg, 2000). The introduction of amino-allyl modified bases (Langer et al., 1981) allowed the chemical synthesis of multiply-labeled fluorescent oligomer hybridization probes (Femino et al., 1998; Kislauskis et al., 1993). This, in turn allowed the application of multicolor/multi-spectral FISH (Nederlof et al., 1990) to visualization of multiple RNA species simultaneously (Levsky et al., 2002). With the introduction of visualization to gene expression assays we begin to understand the complexity of behavior at the cell level, allowing re-investigation of assumed consistencies of cell populations with single-cell resolution (Elowitz et al., 2002; Levsky et al., 2002).

II. Materials and Instrumentation

Oligomer probes were designed with OLIGO software from Molecular Biology Insights, and synthesized on an Applied Biosystems automated DNA/RNA synthesizer (Model 392/394). Solid-support synthesis columns were from Applied Biosystems (dA, Cat. No. 400949; dC, Cat. No. 400950; dmf-dG, Cat. No. 401184; T, Cat. No. 400952). Phosphoramidites (dA, Cat. No. 10-1000-10; dC, Cat. No. 10-1010-10; dmf-dG, Cat. No. 10-1029-10; dT, Cat. No. 10-1030-10) and an amino-allyl modified base for attachment of ester-conjugated fluorophores (C6-dT, Cat. No. 10-1039-05) were obtained from Glen Research. Oligonucleotide Purification Cartridges (OPCTM, Cat. No. 400771) and 2M triethylamine acetate (TEAA, Cat. No. 400613) were from Applied Biosystems and the anhydrous acetonitrile (Cat. No. 40-4050-50) was from Glen Research. Trifluoroacetic acid (TFA, Cat. No. BP618-500) was from Fisher and triethylamine (TEA, Cat. No. T-0886) was from Sigma.

Fluorophores were purchased from Amersham (Cy3, Cat. No. PA23001; Cy3.5, Cat. No. PA23501; Cy5, Cat. No. PA25001) and Molecular Probes (Oregon Green 488, Cat. No. O-6147; Alexa Fluor 488, Cat. No. A-10235 or A-20191). Sodium bicarbonate for labeling buffer (Cat. No. BP328-1), 25-ml pipets for make-shift size-exclusion chromatography columns (Cat. No. 13-674-41E), and Pasteur pipettes (Cat. No. 13-678-20D) for filling the columns were from Fisher. Sephadex G-50 resin (Cat. No. G-50-150) for purification was from Sigma. Columns were fashioned by removing the cotton from the top of one of these pipets and using a portion of it to plug up the tip. Secure the “column” vertically to a ring stand and cap the tip with a 1.5 ml Eppendorf tube to prevent liquid loss. The vacuum concentrator system used was from Savant (Speed-Vac), and the UV-Spectrophotometer used for measurements of probe concentrations and labeling efficiencies was from Beckman (DU640).

Glass cover slips (Cat. No. 12-542B), glass slides (Cat. No. 12-518-103), 12N HCl (Cat. No. A144-212), gelatin (Cat. No. G8-500), parafilm (Cat. No. 13-374-12), forceps for coverslip manipulation (Cat. No. 08-953-E) and magnesium chloride (Cat. No. BP214-500) were purchased from Fisher. 20% paraformaldehyde for preparation of fixative (PFA, Cat. No. 15713) and coplin jars for washes (Cat. No. 72242-01) were from Electron Microscopy Sciences. 10X phosphate buffered saline (PBS, Cat. No. 1 666 789), 20X sodium chloride / sodium citrate (SSC, Cat. No. 1 666 681), purified bovine serum albumin (BSA, Cat. No. 711 454) and E. coli tRNA (Cat. No. 109 541) were from Roche. Triton X-100 (Cat. No. T-9284), formamide (Cat. No. F-4761), sheared salmon sperm DNA (ssDNA, Cat. No. D-7656), 4’,6-diamidino-2-phenylindole (DAPI, Cat. No. D-8417), and diethylpyrocarbonate (DEPC, Cat. No. D-5758) were obtained from Sigma. The glass plates used for hybridization (Cat. No. 165-1824) were obtained from Bio Rad. The ProLong Antifade Kit for mounting slides (Cat. No. P-7481) was from Molecular Probes.

Upright fluorescence microscopes from Olympus were used to image multiple spectral signatures from the FISH specimens (models AX70 and BX51) with a piezoelectric translator from Physik Instrumente (Cat. No. PZ54 E) to generate three-dimensional image stacks. Alternatively, microscopes featuring an internal harmonic drive may be used (e.g. BX61 from Olympus). Illumination was provided by a 100 W Mercury arc lamp. Microscopes were outfitted with Olympus PlanApo 60x, 1.4 NA objectives and Chroma HiQ band pass filters to separate fluorescence signals. Although other methods have been introduced to discern multiple fluorescence signals from chromosomes (Schrock et al., 1996), they have not been successfully applied to detection of mRNA transcription sites. In Levsky (2002) we performed color coding of transcripts using the following filters from Chroma: DAPI (Cat. No. 31000), FITC (Cat. No. 41001), Cy3 (Cat. No. SP-102v1), Cy3.5 (Cat. No. SP-103v1) and Cy5 (Cat. No. 41008). High-resolution, low-noise fluorescence images were captured using Charge-Coupled Device (CCD) cameras from Roper Scientific (models CH-350(502) and CoolSNAP-HQ). Acquisition and data manipulation were performed using IPLab software from Scanalytics. To ease data processing and avoid manual manipulations that introduce bias, we coded our own filtering and data analysis software in the JAVA Programming Language using the Java Development Kit and Advanced Imaging Library from Sun Microsystems.

III. Procedures

A. Preparation of Fluorescent Oligomer Hybridization Probes
This procedure is according to Kislauskis (1993).

Solutions

- 1. Diethylpyrocarbonate-treated distilled water (DDW): To make 1 liter, add .5 ml DEPC to 1 liter of distilled water. Shake or stir until DEPC is well distributed and then autoclave. Prepare enough of this to use in all other solutions.
- 2. Labeling buffer (0.1 M Na2CO3 sodium carbonate, pH 9.0): To make 100 ml, weigh 1.06 g Na2CO3 and complete to 100 ml with DDW. Adjust pH to 9.0 by adding 10N NaOH and store at 4°C.
- 3. 2M TEAB stock: To make 500 ml, take 138.3 ml (101 g) TEA and fill to 400 ml with DDW. Use dry ice to bubble in CO2 until pH is below 8.0. Complete to 500 ml with DDW and store at 4°C. *TEA is extremely hazardous so take care when handling. Use glassware instead of plasticware when measuring and transporting.
- 4. Filtration column running buffer (10mM TEAB): To make 1 liter, take 5 ml of 2M TEAB stock solution and complete to 1 liter with DDW. Store at 4°C.
- 5. Filtration column running matrix: To make approximately 200 ml, pour 200 ml of 10 mM TEAB into an Erlenmeyer flask. Add approximately 5 g of Sephadex G-50 and swirl to absorb the liquid. Suspension will settle. Store at 4°C. Prior to use, apply vacuum pressure to the flask to degas the suspension for at least 2 hours before pouring matrix.

Steps

- 1. Having selected a gene of interest, choose four to five regions for probe fabrication, each 50 bases in length. Adjust search parameters within the OLIGO software to receive best possible sequences for gene detection. Several considerations for probe design are:
    - i. Spanning different areas of the mRNA increases chances detection; intronic regions should be avoided.
    - ii. 50% GC content (or close to this) is optimal.
    - iii. Highly stable hairpins should be avoided.
    - iv. There must be enough well-spaced residues for substitution of modified bases. This depends on the modifier used. We spaced five modified thymidine residues at 8 or more bases apart.
    - v. The sequences must not cross-react significantly with other mRNAs. Use BLAST to test this (see http://ncbi.nih.gov/blast).
- 2. Prepare the reversed antisense sequence of each designed oligo, substituting the modified bases appropriately.
- 3. Synthesize the oligos according to synthesizer specifications at a 0.2 µM scale, specifying TRITYL-ON.

  Deprotect the crude products in a 65°C water bath for one hr.
- 4. Aliquot the crude product into 200-300 µl portions and set one aside for immediate purification. Vacuum-dry the remaining aliquots, then re-suspend each pellet in 1.0 ml 10 mM TEAB plus 5 µl TEA. Store these at –80°C for future use. *As the aliquots dry, the solutions become increasingly acidic and may cause detritylation of the oligos. To avoid this, add a drop of TEA to each tube periodically while drying them.
- 5. Purify the remaining aliquot using the OPCTM according to recommended procedures (Applied Biosystems). Vacuum-dry the final pure product, then re-suspend in 50 ml DDW. Determine concentration of product using OD measurements at 260 nm.
- 6. Prepare a probe mixture with equal amounts of each oligo to obtain a final amount of 20 µg – either 4 or 5 µg of each oligo depending on how many were synthesized. Vacuum-dry this mixture.
- 7. Resuspend the pellet in 10 µl labeling buffer, and add it to the reaction vial containing approximately 1.0 mg of dye-ester conjugate. Alternatively, oligos can be labeled according to manufacturer's specifications (Amersham or Molecular Probes). Vortex and leave at room temperature overnight.
- 8. Assemble a size-exclusion chromatography column by transferring 10 mM TEAB via glass Pasteur pipet into the prepared 25 ml pipet/column until liquid level is about a third of the way up. Add the G-50 suspension in the same manner and, as the matrix settles, remove the 1.5 ml tube "cap" to allow the matrix to settle above the cotton-plug stop, while permitting liquid to pass through. After the matrix has filled the pipet pack it down with a continuous flow of TEAB for 10-15 min. This can be most easily accomplished by using a siphoning system attached to the column.
- 9. Once the matrix has packed, remove the siphoning attachment and allow the buffer to run down to the level of the matrix, taking care not to let it run below. Add the 10 µl volume of probe/dye mixture to the column and wash the reaction vial with an additional 200-300 µl of fresh TEAB. Add the wash to the column and allow it to begin to run down into the matrix. When the dye product has been absorbed into the matrix, refill the column with buffer and reattach the siphoning system to provide continuous liquid flow.
- 10. As the labeled probe mixture runs down the column it will separate into two bands. The first, faster band will contain the desired pure product. Collect column eluates in 1.0 ml fractions to include this first band. Vacuum-dry these fractions. Re-suspend the selected fractions in DDW to achieve a total volume of 500 µl.
- 11. Measure OD of the final sample to determine final concentration and labeling efficiency for the product

according to specifications of the dye manufacturers (Amersham, Molecular Probes). A final concentration of 40 ng/µl would indicate that all 20 µg of oligo initially labeled has been collected.

- 12. Labeled probe can be stored at 4°C, or at –20°C for longer term storage.

B. Preparation of Cell Samples

Solutions

- 1. Coverslips in 0.5% gelatin: To make 200 ml, sterilize a box of coverslips by boiling in 0.1 N HCl for 20 min. Rinse and wash the coverslips in DDW several times. Weigh 1.0 g of gelatin and complete to 200 ml DDW. Stir and warm to dissolve completely. Transfer sterilized coverslips to gelatin solution and autoclave for 20 min. Store at 4°C.
- 2. 10X PBS stock: To make 500 ml of DEPC-treated 10X PBS, take 500 ml 10X PBS and add 250 µL DEPC. Stir or shake to dissolve; autoclave.
- 3. 1 M MgCl2 stock: To make 100 ml, weigh 20.3 g MgCl2 and complete to 100 ml with DDW.
- 4. Washing solution (PBSM): To make 1 liter, take 100 ml 10X PBS stock, add 5 ml 1 M MgCl2 stock and complete to 1 liter with DDW.
- 5. Extractant (PBST): To make 1 liter, take 100 ml 10X PBS stock, add 5 ml Triton X-100, and complete to 1 liter with DDW. Stir gently to allow Triton to dissolve completely. *This extractant has been used successfully to remove cytoplasm in cultured DLD-1 cells. The strength of the extractant must be optimized for each cell type to obtain optimal reduction of cytoplasmic background without damaging nuclei or loss of cells.
- 6. Fixative (4% PFA): To make 50 ml, take one 10 ml vial of 20% paraformaldehyde stock, add 5 ml 10X PBS stock, and complete to 50 ml with DDW. Store at 4°C.

Steps

- 1. Grow cells under standard conditions and seed onto gelatinized cover slips in a petri dish. Cells are grown to empirically-determined confluence such that they are sparse enough to facilitate automated separation of nuclei during image processing, and dense enough to have significant amounts for analysis.
- 2. Any treatment steps, such as serum starvation and stimulation, can be performed at this point before fixation.
- 3. Wash the cells briefly with ice-cold PBSM.
- 4. Extract the cells for 60 seconds in PBST at room temperature.
- 5. Wash the cells twice briefly with ice-cold PBSM.
- 6. Fix the cells with the PFA fixative solution for 20 min at room temperature.
- 7. Wash the cells again twice briefly with ice-cold PBSM.
- 8. Fixed cover slips may be stored at 4°C in PBSM until use. *Further extraction and background reduction can be obtained for some cell types by storage in 70% ethanol at 4°C. In some cases this can cause cells to detach from cover slips.

C. Hybridization

This procedure is modified from Femino (1998) and Levsky (2002).

Solutions

- 1. Washing solution (PBSM): To make 1 liter, take 100 ml 10X PBS stock, add 5 ml 1 M MgCl2 stock and complete to 1 liter with DDW.
- 2. Pre/post-hybridization wash (50% formamide/2X SSC): To make 500 ml, take 250 ml formamide, add 50 ml 20X SSC stock and complete to 500 ml with DDW.
- 3. Probe competitor solution (ssDNA/tRNA): To make 100 µl of 10 mg/ml total concentration competitor, take 50 µl of 10mg/ml sheared salmon sperm DNA and add 50 µl 10mg/ml E. coli tRNA (prepared from solid by adding 10 mg to 1.0 ml DDW). Store at –20°C.
- 4. Hybridization buffer: To make 100 µl, take 60 µL DDW and add 20 µL BSA, and 20 µL 20X SSC stock. Prepare fresh and hold on ice. *This volume is sufficient for 10 hybridization reactions (10 coverslips).
- 5. Low-salt wash solution (2X SSC): To make 500 ml, take 50 ml 20X SSC stock and complete to 500 ml with DDW.
- 6. Nuclear stain solution (DAPI): To make 1 liter, take 100 ml 10X PBS stock, add 50 µL 10 mg/ml DAPI stock (prepared from solid by adding 10 mg to 1.0 ml DDW) and complete to 1 liter with DDW. Shake or mix to dissolve DAPI completely and store at 4°C.
- 7. Mounting medium: To make 1 ml, prepare ingredients of ProLong kit according to manufacturer's specifications (Molecular Probes) or use an equivalent method. About 25 µl of medium is needed per cover slip.

Steps

- 1. Hybridization is tested before color-coding and multiple transcript detection. We started by using two bright dyes (Cy3 and Cy5) to show transcription sites. After this, each gene is assigned an arbitrary color code using combinations of dyes and tested singly. Only after results are reproducible is multiplex detection

code using combinations of dyes and tested singly. Only after results are reproducible is multiplex detection performed.

- 2. Using forceps, place fixed coverslips vertically in a coplin jar, keeping note of which side has the cells on it. Rehydrate and wash the cells in PBSM for ten min. *All washes are at room temperature unless otherwise noted.
- 3. Equilibrate the cells in pre-hybridization solution for ten min.
- 4. Aliquot probe mixtures for gene(s) to be detected into tubes for each different combination of targets to be assayed. *As a starting concentration, combine 20 ng of each probe for the 20 µl total final reaction volume. Optimal concentrations of the different probe mixtures are determined empirically by balancing the resultant colors detected upon imaging transcription sites.
- 5. Add competitor solution to the probe mixture(s) in 100-fold excess. Vacuum-dry this mixture, taking care not to over dry.
- 6. Re-suspend the dry pellet in 10 µl formamide and place the tubes on a heating block at 85°C for 5-10 min, then place immediately on ice.
- 7. Add 10 µl of hybridization buffer to each tube, giving a final reaction volume of 20 µl.
- 8. Wrap a glass/plastic plate with parafilm to allow enough working space for the amount of reactions you have. Dot each 20 µl reaction volume onto the plate, far enough apart such that cover slips can be placed over each volume without overlap.
- 9. Remove cover slips from pre-hybridization solution and blot off excess liquid. Place each cover slip – cell side down – on the hybridization mix already dotted onto the plate.
- 10. Wrap another layer of parafilm over the plate and cover slips to seal the reactions. Press around the edges with a pen or similar instrument.
- 11. Incubate the plate at 37°C for three hr, along with a sufficient amount of pre-hybridization solution to wash the cover slips twice after hybridization.
- 12. Remove the top layer of parafilm and carefully lift the lower layer so that the cover slips can be removed easily without excessive manipulation. Place the cover slips back into coplin jars with the pre-warmed wash – keeping track of the cell-side – and incubate for 20 min at 37°C. Change and repeat this wash for another 20 min.
- 13. Change the solution with 2X SSC and incubate at room temperature for ten min.
- 14. Change the solution with PBSM and incubate at room temperature for ten min.
- 15. Counterstain the nuclei by changing the solution with the prepared DAPI and incubating at room temperature for one min, then washing with PBSM.
- 16. Change the PBSM and keep at room temperature until ready to mount.
- 17. Mount each coverslip (cell-side down) onto glass slides, using freshly prepared antifade mounting medium. Blot off excess liquid and store at –20°C.

D. Microscopy and Image Analysis
These procedures are from Levsky (2002).

Steps

- 1. Image stacks are acquired with high index oil immersion on a fluorescence microscope outfitted for optical sectioning. We used a step size of .5 µm to generate image volumes as transcription site signals are bright and did not require more finely spaced planes on our setup. For future processing steps and for detecting less bright signals, closer optical sections may be needed. We used the 60X objective and additional magnification (when necessary) to yield digital images of roughly 100 nm-per pixel resolution. The total magnification should be adjusted to yield similar resolution given the physical size of elements on the CCD camera used. High resolution enables morphometric processing of the signals.
- 2. Image volumes from different fluorescent channels are normalized by contrast enhancement to ensure interpretation is independent of relative intensity. This can be performed with commercial software, such as IPLab (Scanalytics), for the entire three-dimensional image stacks at one time to ensure that the sample is analyzed evenly. The ‘black value’ for the enhancement should be set to the approximate extra-nuclear noise level for the sample, which can vary markedly. The ‘white value’ should be slightly above the intensity for the center of the brightest signals, namely nuclear sites of transcription.
- 3. Digital signal enhancement can be approached by two methods – direct analysis of three-dimensional images or splitting the image into two-dimensional slices, slice-by-slice processing, and finally, collation of the data into a three-dimensional representation. Both approaches require similar filtering algorithms, but currently-available implementations generally require decomposition into slices as they can only process two-dimensional images. Either way, the basic method of signal enhancement is simple convolution filtering using a kernel that approximates the size of the target signal. This implies that the kernel should be adjusted to approximate the size of empirically observed sites of transcription, as determined by the magnification used in image acquisition. The designed kernel should include surround penalty to decrease the chances of false-positive detection of larger areas of fluorescence noise (intrinsic or extrinsic to the sample). The center, or positively scored part of the kernel, should be large enough to ignore specular noise and camera defects, which can appear as highly intense single pixels.

- 4. Positive detection of sites of transcription depends on empiric selection of a threshold. If contrast enhancement (step 1) was performed correctly, this should allow a single color level to be used to distinguish between background levels and transcription site color codes in all fluorescence channels. This procedure may be performed using a segmentation algorithm for each color combination used for detection in the image, such that one singles out sites of each identity one at a time. Finding this tedious, we prefer to detect all supra-threshold signals and determine the color-coded identity at once by coding a simple algorithm. This procedure involves scanning the image pixel by pixel for supra-threshold signal, recording each putative signal, and marking off contiguous regions surrounding the signal such that they are not scored more than once. Location of the signal and intensity in all color bands (both point-wise and with surrounding-area) are recorded. The intensity values are compared with the threshold and identity of the site is assigned.
- 5. For visualization purposes, a pseudo-colored, flattened two-dimensional rendering is prepared. For a background, we prefer to use the middle Z-slice of the nuclear counter-stain image. Transcription site locations and identities (now arbitrarily pseudo-colored and depicted with an artificial marker in the image) are shown. We have added a number adjoining the site to mark the Z-section from which the center of the site was detected. This is necessary since the filtered and threshold-corrected data contains more than three colors of images and cannot be depicted unambiguously in red-green-blue color systems.
- 6. Nuclear bounds are generated by binarization and simple flood-fill of the nuclear counter-stain image. Binarization requires a single threshold to be chosen to distinguish between intra-nuclear and extra-nuclear; this can usually be done given appropriate exposure of the counter-stain. Flood-fill algorithms will only work with discretely separated nuclei and must be modified significantly to interpret overlapping signals. Nuclei for which the flood-fill-defined area extends to the edge of the image plane should be ruled out for further analysis as their contents are incompletely imaged.
- 7. Joining the results of steps 4 and 5 now yields the data of single cell gene expression profiles – a set transcription sites for each nucleus analyzed. Each transcription site detected in a field is placed onto the flood-fill map and assigned a nucleus. Sets of nuclear data are exported for further statistical study.

IV. Pitfalls

- 1. The overlap of fluorophore colors should be carefully considered when designing a barcoding scheme. Consideration of the strength of fluorophores, the separation between emission spectra, excitation characteristics of the lamp, and the filter sets to be used to discern signals is critical.
- 2. When assembling the G-50 column and loading sample do not let the liquid level run below the matrix. This will create cracks and bubbles, potentially disrupting complete band separation and adding to contamination of product with free dye.
- 3. Poorly labeled probes (< 40%) can fail to detect transcription sites. To increase labeling efficiency, multiple serial labelings and purifications can be performed.
- 4. Probe mixtures that have a suspiciously high level of labeling (> 80%) may contain free dye, which will increase background. Multiple purifications by G50 column can be used to remedy this.
- 5. When placing the cover slips down onto the parafilm-coated plates, care should be exercised to avoid bubbles occurring and thereby preventing total contact of the probe with the cover slip. Also take care not to touch or move the cover slips excessively once they are placed onto the parafilm as this may contribute to cell detachment and damage.
- 6. Some cell types have high inherent autofluorescence obscuring nuclear signals. Careful processing with adequate extraction can remedy this at times. Additional processing steps may be necessary for recalcitrant noise problems.
- 7. Transcript color codes in which the colors are inadequately balanced may ‘decay’ such that the observed signal is misinterpreted as a different color code containing a subset of the original code. This is especially problematic under conditions of low transcript abundance and with less intense fluorophores (like the FITC-derivatives). Color codes must be carefully tuned before multiplex detection.

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