Flow Sorting from Organ Material by Intracellular Markers

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Background: Fluorescence-activated cell sorting (FACS) is an attractive technique for gene or protein expression studies in rare cell populations. For cell types where specific surface markers are not known, intracellular markers can be used. However, this approach is currently held to be difficult, as the required fixation and permeabilization may cause protein modification and RNA degradation.

Methods and Results: Using the rat thyroid gland as model, rare (parafollicular) and frequent (follicular) endocrine cell types were sorted based on immunostaining for intracellular calcitonin peptide and thyroglobulin protein expression. The sorted cells were compatible with Western blot analysis of proteins, immunoassay detection of calcitonin peptide hormone and RT-PCR.

Conclusion: We developed a robust FACS protocol that allows flow sorting of rare cells from dissociated organ material, based on intracellular markers. Our FACS protocol is compatible with downstream analysis of proteins, peptides, and mRNA in the sorted cells. © 2007 International Society for Analytical Cytology

Key terms: intracellular staining; FACS; thyroid; RT-PCR; western blot

In the living animal, rare cell subsets can have important regulatory functions, for example the calcitonin-producing C-cells (parafollicular cells) in the thyroid gland. It would be desirable to have general methods for purification of rare cells from organ material, for molecular studies of protein and mRNA expression. Fluorescence-activated cell sorting (FACS) is a general method for isolating cells labeled with fluorescent tags. However, flow sorting cells from organ material requires dissociation of the tissue into single-cell suspension using proteases, a treatment that may destroy surface markers. Also, many important rare cell subtypes are best characterized by cytoplasmic protein or peptide hormone products, for example calcitonin in C-cells, or insulin in β cells of pancreatic islets. Thus, sorting from organ material based on intracellular markers would in many cases be an attractive option. Unfortunately, the fixation and permeabilization required for intracellular staining can be associated with RNA degradation (1–3).

Reflecting the technical difficulties, only few studies have described molecular analysis of cells flow-sorted for intracellular protein markers (1–3). Most work has been done on cell lines (1,2), with only one study describing flow sorting from dissociated biopsy specimens (3). In that study, an abundant cell population was sorted (epithelial cells from mammary biopsies), and only RT-PCR was performed on the sorted cells (3). Thus, very little technical information is currently available regarding flow sorting from organs by intracellular markers, especially as regards rare cell subsets, and protein and peptide analysis from the sorted cells.

Therefore, in the present work, we developed a simple FACS protocol for flow sorting rare cells from organs by intracellular markers. Our protocol implements simple remedies at critical steps, to ensure RNA, protein, and peptide integrity in the sorted cells. Using the rat thyroid gland as model, we show that the protocol is compatible with subsequent molecular characterization of protein, peptide hormone, and mRNA expression of even low-abundance targets in the sorted cell populations.

MATERIALS AND METHODS
Dissociation of Thyroid Glands into Single-Cell Suspensions

Dissociation of thyroid glands into single-cell suspensions was adapted from (4). Male Wistar rats (above 6 months old) were anesthetized with isoflurane/N_2O and euthanized by severing the abdominal aorta. The thyroid glands were removed and placed in ice-cold (4°C) minimal essential medium (MEM) (Gibco, Denmark) supplemented with 50 μg/ml gentamicin (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The intact thyroid glands were rinsed in ice-cold dissociation medium [MEM supplemented with 0.5 U/ml collagenase II (Gibco), 1.2 U/ml Dispase (Gibco), 100 μg/ml DNase (Roche, Denmark), 1 vol % heat-inactivated fetal calf serum, 50 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco)], and transferred into non-cell-binding 60-mm petridishes (Corning, UK) in a small volume of dissociation medium, sufficient to keep the tissue moist. The thyroids were minced using a scissor, and incubated in dissociation medium at 4°C overnight. Next day, the minced tissue was transferred into sterile tubes, added 2–3 volumes fresh dissociation medium, and incubated for 2 h on a 37°C water bath with magnet stirring. The minced thyroid tissue was quite sticky, and larger tissue clumps invariably formed during the 2-h incubation; these were dissociated with sterile scissors at regular intervals. Typically, glands from up to 10 animals were dissociated in a total of 20-ml dissociation medium. The dissociated tissue was filtered through a 200-μm nylon mesh (DAKO Glostrup, Denmark), and the cells were pelleted at 300g for 7 min at 4°C and the supernatant was discarded. The cell pellet was resuspended gently but thoroughly in the residual fluid volume (∼100 μl), and 10 volumes (1 ml) of 4°C methanol was added, followed by brief vortexing. Cells in methanol were stored at −20°C. This fixation, permeabilization, and storage method was adapted from Krutzik and Nolan (5).

Antibodies

FITC-conjugated monoclonal antibody against human thyroglobulin was purchased from abcam (UK) (murine IgG1, ab8571, clone B34.1). This antibody recognizes unprocessed as well as processed thyroglobulin (330–60 kDa) (6). FITC-conjugated antirat CD45 was used as an isotype-matched negative control (murine IgG1, BD 554877). Polyclonal rabbit antihuman calcitonin immunoglobulin (DAKO Denmark; A5076) was phycoerythrin-conjugated using the AnaTag™ R-PE Protein Labeling Kit (Nordic Bio-Site, Stockholm, Sweden). To control for calcitonin-staining specificity, 80 μg/ml anticalcitonin antibody was incubated with 2 μg/ml human calcitonin peptide (Sigma, Denmark) for 30 min at room temperature, and then used for staining. Western analysis of GAPDH (MW 36 kDa), β actin (MW 42 kDa), and α tubulin (MW 50 kDa) was done with monoclonal antibodies from abcam and Sigma (ab8245, ab6276 and T6199).
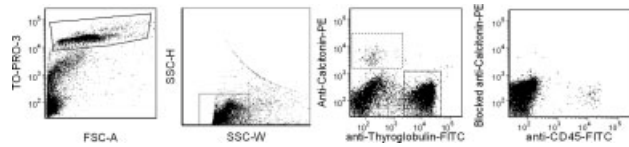

FIG. 1. Gating strategy for sorting of triple-stained, enzyme-dispersed rat thyroid gland cells. **Left Panel**: TO-PRO-3 sorting gate, defining nucleated cells. This is also the sorting gate used for "thyroid cells." Events outside the gate were defined as debris. **Middle Left Panel**: Gated TO-PRO-3 nucleated cells are shown. The SCC-W/SSC-H sorting gate was used to define singlets, and exclude doublets and aggregates. **Middle Right Panel**: Events double-gated for singlet nucleated cells were resolved as thyroglobulin positive (follicular cell sorting gate, dotted line) or calcitonin positive (C cell sorting gate, dashed line). "Non C-/follicular cells" were defined as the population with low FITC and PE fluorescence, outside the C cell and follicular cell sorting gates. **Right Panel**: Negative control sample, thyroid cells stained with calcitonin peptide-blocked anticalcitonin antibody and thyroglobulin isotype control (rat anti-CD45). FSC and SSC, linear scales. PE and FITC fluorescence, logarithmic scales.

FACS

Methanol-fixed and permeabilized cells were washed twice in 0.2-μm sterile-filtered FACS buffer: PBS (RNase-free, Ambion, UK) supplemented with 0.5% bovine serum albumin (Sigma), 0.05% sodium azide, and, for RT-PCR experiments, also 10 μg/ml yeast tRNA (Ambion). The cells were incubated with 1 μg/ml FITC-conjugated antithyroglobulin monoclonal antibody and 0.8 μg/ml PE-conjugated rabbit anticalcitonin immunoglobulin for 1 h on ice, in the dark. Negative controls consisted of FITC-conjugated antirat CD45 monoclonal antibody, and calcitonin peptide adsorbed PE-conjugated rabbit anticalcitonin immunoglobulin. Usually, for cells from 30 to 45 rats, the staining volume was 1 ml. After antibody incubation, TO-PRO-3 (Molecular Probes, Denmark) was added to 1 μM final concentration, to allow discrimination between nucleated cells and debris. Cells were washed once in ice-cold FACS buffer, and sorted on a FACS Vantage SE with the DiVa option. The FACS instrument was not cleaned in any special way. Debris was excluded by gating on TO-PRO-3 positive events, and doublets were excluded using SSC-W/SSC-H plots. Sorting gates are shown in Figure 1. In total, TO-PRO-3 positive cells were sorted into four separate populations: C cells (calcitonin positive cells), follicular cells (thyroglobulin positive cells), non C-/follicular cells (calcitonin and thyroglobulin negative cells), and thyroid cells (all TO-PRO-three positive events, excluding debris). Cells for RT-PCR were sorted into 200 μl RNA later (Ambion). After sorting, the cells were pelleted, lysed, and used for either RT-PCR or immunoassay and Western blotting analysis.

RNA Extraction and Quantitative RT-PCR

Cell pellets were lysed by freeze/thawing in a guanidine isothiocyanate lysis buffer (5 M guanidine isothiocyanate: 0.5% N-lauryl sarcosine: 32 mM citrate, pH 5.2) (GuSCN lysis buffer). Total RNA was extracted by acid phenol/1-bromo-3-chloropropane followed by binding of the RNA to silica particles, as previously described (7,8).

Table 1
Description of PCR Primers and TaqMan Probes

Gene target	GenBank accession number	Primer and probe nucleotide sequence	Final concentration in PCR reaction (nM)
Calcitonin	NW_047562	Forward primer 5′ AGGAGGCTGAGGGCTCTAGCT 3′	900
		Reverse primer 5′ CCCAGCATGCAGGTACTCAGA 3′	900
		Probe 5′ ACAGCCCCAGATCTAAGCGGTGTGG 3′	250
GAPDH	NW_043769	Forward primer 5′ CATGGCCTTCCGTGTTCCTA 3′	300
		Reverse primer 5′ CCTGCTTCACCACCTTCTTGAT 3′	900
		Probe 5′ CCGCCTGGAGAAACCTGCCAAGTATG 3′	250
β Actin	NW_042778	Forward primer 5′ CACAGCTGAGAGGGAAATCGT 3′	300
		Reverse primer 5′ TGGATGCCACAGGATTCCAT 3′	300
		Probe 5′ ATGGCCACTGCCGCATCCTCTTC 3′	250
HPRT-1	NM013556	Forward 5′ CAGCCCCAAAATGGTTAAGGT 3′	900
		Reverse primer 5′ AACAAAGTCTGGCCTGTATCCAA 3′	900
		Probe 5′ AAGCTTGCTGGTGAAAAGGACCTCTCGAA 3′	250
18S		Eukaryotic 18S rRNA Endogenous Control kit (Applied Biosystems)	

Oligonucleotides were synthesized by DNA Technology A/S, Aarhus, Denmark. The TaqMan probes were fluorochrome-labeled following standard recommendations from Applied Biosystems (5′ FAM, 3′ TAMRA). The eukaryotic 18S rRNA Endogeneous Control probe was labeled with VIC and TAMRA fluorochromes at the 5′ and 3′ ends, respectively (Applied Biosystems).

First strand cDNA synthesis was performed using the Retroscript™ Kit according the manufacturer's instructions (Ambion). Briefly, each reaction contained 10 μl RNA, 1x RT buffer, 500 μM of each dNTP, 5 μM random decamers, 100 U MMLV reverse transcriptase, and 10 U placental RNase inhibitor, in a total volume of 20 μl. The RT reactions were incubated for 1 h at 44°C, and heat-inactivated at 92°C for 10 min. Real-time PCR was performed in 25 μl reactions containing: 1 μl cDNA, 1× TaqMan Universal PCR mastermix (Applied Biosystems, Denmark), 0.5 U AmpErase® Uracil *N*-Glycosylase (UNG) (Applied Biosystems), and gene-specific primers and TaqMan probes (Table 1). To minimize the risk of nucleotide polymorphisms in primer and probe regions affecting quantitation, the PCR primers and TaqMan probes were all designed to be located in regions conserved between rat and mouse. The TaqMan assays were intron-spanning, to exclude amplification of genomic DNA. Thermal cycling was done using an Abi Prism® 7000 Sequence Detection System cycler under the following conditions: [50°C for 5 min], [95°C for 10 min], 45× [95°C for 15 s, 60°C for 30 s, 72°C for 30 s], [72°C for 5 min]. Calcitonin mRNA levels were quantitated relative to the reference gene GAPDH, as recommended by the reagent manufacturer (Applied Biosystems 2001).

Calcitonin Immunoradiometric Assay

Sorted cells were pelleted, the supernatants removed, and the cell pellets vortexed to loosen the cells. Cells were lysed by adding 100 μl Western blot lysis buffer and incubating at 70°C for 15 min. The Western blot lysis buffer consisted of NuPAGE LDS buffer (Invitrogen, Denmark), 5 mM EDTA, 1/10 volume reducing agent (Invitrogen), and 1/100 volume protease inhibitor cocktail III (Calbiochem, UK).

Lysed samples were diluted 125× in PBS supplemented with 0.1% BSA and 0.05% Tween-20. Absolute calcitonin levels were determined using a quantitative rat calcitonin immunoradiometric assay, following the manufacturer's instructions (Immutopics, San Clemente, CA, USA).

Western Blot Under Fully Denaturing and Reducing Conditions

Sorted cells were lysed, reduced, and heat-denatured in Western blot lysis buffer, as described above for the IRMA assay. Electrophoresis was performed on precast 4–12% gradient NuPAGE gels (Invitrogen), followed by transfer in 1× transfer buffer with 10% methanol to 0.45 um PVDF membranes for 1 h at 30 V (reagents and transfer units from Invitrogen). The membranes were blocked with 5% skimmed milk in PBS with 0.1% Tween-20 (BLOTTO), and incubated with primary antibody dilutions for 1 h at room temperature. Antihuman thyroglobulin monoclonal antibody was diluted 1:1,000 in BLOTTO. A cocktail of monoclonal antibodies against three housekeeping proteins was made by diluting antihuman GAPDH, antihuman actin, and antihuman tubulin at 1:100,000, 1:80.000, and 1:6,000, respectively, in BLOTTO. Following incubation with primary antibodies, the membranes were washed for 1 h in several changes of PBS with 0.1% Tween-20. Then, the membranes were incubated with HRP-conjugated goat anti-mouse immunoglobulin (Cell Signaling) at 1:10,000 in BLOTTO, and washed as described above. Protein bands were visualized using ECL[Advance] chemiluminescent substrate (GE Healthcare, Denmark) and a CCD camera (LAS3000, FujiFilm, Sweden).

RESULTS AND DISCUSSION
Antihuman Antibodies Cross-React with Rat Thyroglobulin and Calcitonin

The thyroid gland contains two endocrine cell populations, parafollicular (C cells) and follicular cells. C cells and follicular cells are characterized by unique intracellular products, calcitonin and thyroglobulin, respectively

(9). Rat thyroid glands were dissociated by enzymatic treatment, and the resulting cell suspensions were fixed and permeabilized with methanol, and stained with anti-human calcitonin and thyroglobulin antibodies. To allow specific examination of nucleated thyroid cells and exclusion of debris during sorting, TO-PRO-3 staining was used (10). The antihuman calcitonin antibody stained a proportion of rat thyroid cells commensurate with the described C cell frequency in this species (11). The staining could be abrogated by preincubating the antibody with human calcitonin peptide (Fig. 1). Thus, the antihuman calcitonin antibody could be used as a specific probe for rat C cells, as expected based on the high degree of conservation between rat and human calcitonin (30 of 32 aminoacid identity). Similarly, the antihuman thyroglobulin antibody specifically stained rat follicular cells, as evidenced by comparison to an isotype-matched negative control antibody (rat α-CD45).

Using this approach, we consistently found that in 6-month-old rats, enzymatically digested thyroid tissue contained ~40% follicular cells and ~2–4% C cells (Fig. 1). The remaining population, constituting ~60% of thyroid cells, was not examined further, but probably contained parathyroid, endothelial, blood cells, and other stromal cells.

Peptide and Protein Analysis from Sorted Cells

Thyroid cells were flow-sorted into three fractions: calcitonin positive (C cells), thyroglobulin positive (follicular cells), and thyroglobulin and calcitonin negative (non C-/follicular cells) (Fig. 1). Furthermore, as control, TO-PRO-3 cells were sorted into a population designated thyroid cells. The sorted cells were lysed and analyzed for calcitonin and thyroglobulin content by IRMA and Western blot. Calcitonin and thyroglobulin levels were normalized to cell numbers in the sorted populations, as determined by the FACS instrument.

Using this approach, the C cell fraction was shown to contain high levels of calcitonin but no thyroglobulin (Fig. 2B). In contrast, the follicular cells contained thyroglobulin, but no calcitonin (Fig. 2A). The non C-/follicular cell fraction contained neither thyroglobulin nor calcitonin (Figs. 2A and 2B). Finally, the fraction containing all thyroid cell types was shown to contain small amounts of both calcitonin and thyroglobulin (Figs. 2A and 2B).

Using the thyroglobulin antibody for Western blotting, we observed bands at >250 kDa and ~60 kDa (Fig. 2C). The secondary antibody produced an unspecific band at ~100 kDa (Fig. 2C, compare left and right panels). Others have described an apparent molecular weight of 330 kDa for rat thyroglobulin (6). Thus, the >250 kDa band was used to quantify thyroglobulin expression (Fig. 2A). The ~60 kDa band was likely a thyroglobulin processing product, since multiple immunoreactive thyroglobulin processing products have been demonstrated in lysed thyroid cells (12).

The calcitonin and thyroglobulin data showed that the methanol fixation method was compatible with analysis of small peptides as well as large proteins. To further explore
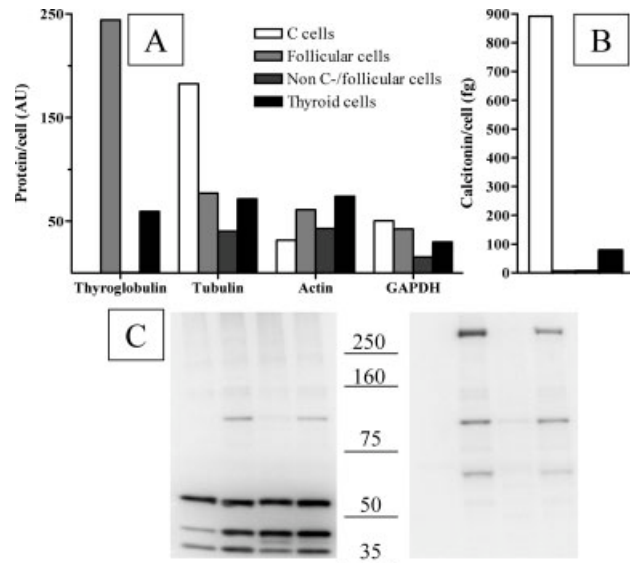

Fig. 2. Protein and peptide analysis on sorted rat thyroid gland cells. Thyroid gland cells were flow-sorted into C cells, follicular cells, non C-/follicular cells, and thyroid cells as described in Materials and Methods and shown in Figure 1. (**A**) Western blot analysis of thyroglobulin and housekeeping proteins (tubulin, actin, and GAPDH) in sorted cells. The band intensities were quantitated using a CCD camera, and normalized to the number of cells in the sorted populations, as determined by the flow sorter instrument. AU, arbitrary units. (**B**) Calcitonin content by IRMA, normalized to the number of cells in the sorted populations. (**C**) The Western blots images used for the quantitative analysis in panel A, showing that the methanol fixation, staining, and sorting did not cause degradation or aggregation of the proteins. (Lanes) 1, C cells; 2, follicular cells; 3, non C-/follicular cells; 4, thyroid cells. (C, Left Western) The blot was probed with a cocktail of three monoclonal antibodies against α tubulin (~50 kDa), β actin (~42 kDa), and GAPDH (~36 kDa). (C, Right Western) The blot was probed with the thyroglobulin antibody that was also used for cell staining prior to FACS. The upper band was used to quantitate thyroglobulin (7), while the middle band was an unspecific band caused by the secondary antibody and present also on the left gel (Fig. 2C, left) and on the secondary antibody only control gel (not shown). The lower band at ~60 kDa is likely an immunoreactive processing product of thyroglobulin (7). For this experiment, the following number of cells was used: C cells, 139,973; follicular cells, 445,974; non C-/follicular cells, 904,085; thyroid cells, 700,000. To obtain these numbers of sorted cells, thyroids from 48 rats were used. In a repeat study, thyroids from 40 rats produced sufficient numbers of sorted cells for a similar analysis.

this for midsize proteins, sorted populations were analyzed for GAPDH, actin, and tubulin. In all cases, nondegraded bands of the expected sizes were observed (Fig. 2C, left). As expected for these housekeeping proteins, levels were roughly similar between the cell fractions, albeit with a tendency for higher tubulin expression in C cells (Fig. 2).

RT-PCR on Flow-Sorted Cells Stained for Intracellular Markers

Next, we wished to address whether RT-PCR could be performed on the permeabilized and sorted cells. In initial experiments, using standard staining protocols, we were not able to detect calcitonin or GAPDH mRNAs by RT-PCR on flow-sorted cells (not shown). This was in accordance with the experience of others (13), and was likely due to RNA degradation during the staining and sorting of the permeabilized cells.

Table 2
RT-PCR Results from Two Independent Sorting Experiments

Cell subset	Number of cells sorted	GAPDH (Ct)	β Actin (Ct)	HPRT-1 (Ct)	18S (Ct)	Calcitonin (Ct)	ΔCt β actin-GAPDH	ΔCt HPRT1-GAPDH	ΔCt 18S-GAPDH	ΔCt Calcitonin-GAPDH
C cells	31,629/96,411	27.0/31.7	34.4/41.6	39.5/39.2	37.6/40.2	32.6/35.9	7.4/9.9	12.5/7.5	10.6/8.5	5.6/4.2
Follicular cells	505,618/660,026	22.4/27.1	30.1/ND	31.1/35.0	30.2/38.3	37.7/41.1	7.6/ND	8.7/7.9	7.8/11.3	15.2/14.0
Non C-/follicular cells	331,670/80,0891	24.3/27.6	31.5/36.7	32.13/33.4	32.2/35.2	38.7/35.5	7.3/9.1	7.9/5.8	8.0/7.6	14.4/7.9
Thyroid cells	120,000/1,006,858	25.1/27.3	32.3/35.7	33.2/33.2	34.4/36.3	ND/35.9	7.2/8.3	8.0/5.8	9.3/8.9	ND/8.6

Results from the two independent sorting experiments are separated by slashes. The number of rats in the two experiments was 40 and 45, respectively.
Ct, threshold cycle value, the PCR cycle number where fluorescence from the TaqMan probes rose above background. Low Ct values indicate high mRNA levels, and high Ct values indicate low mRNA levels, with a difference of 1 Ct value corresponding to a two-fold difference in mRNA abundance, assuming 100% PCR efficacy.
ΔCt values were calculated from the Ct values, as indicated in the table.
ND, not done.

Therefore, our protocol was optimized to reduce RNA degradation. The salient features of the optimized protocol were (see also Materials and Methods): (a) adding yeast tRNA at 10 μg/ml to all buffers, (b) using RNAse-free buffers and water (Ambion), (c) using disposable RNase-free plastware, (d) using filter pipette tips and gloves, (e) keeping the samples on wet ice (0–4°C) and using precooled centrifuges throughout the experiment, (f) using directly conjugated primary antibodies, to reduce the number of steps in the staining protocol, and (g) sorting the cells directly into tubes containing 200 μl of RNA later (Ambion). It should be mentioned here that during optimization of the staining protocol, different RNase inhibitors were tried. We found that ribonucleosid vandyl complexes greatly increased background fluorescence in the cells, and the protein "RNase inhibitor" from Ambion (cat no. 2682) inhibited antibody staining. In contrast, tRNA was compatible with cell staining and FACS, in addition to being very economical in use.

Using the optimized protocol, we were able to RT-PCR amplify mRNAs for high abundance targets such as GAPDH, β actin, 18S rRNA, and calcitonin, as well as low abundance targets such as HPRT-1, from all thyroid cell subsets subjected to FACS (Table 2). RNA degradation is generally assumed to be caused by RNAse contamination from buffers and utensils used in the staining procedure, as well as the fluidics system of flow sorters. While we found that reducing RNase exposure during staining greatly improved the quality of RT-PCR analysis of sorted cells, RNase exposure during sorting may be important in some cases.

To explore the biological validity of the RT-PCR results, we calculated ΔCt β actin-GAPDH, ΔCt HPRT1-GAPDH, and ΔCt 18S-GAPDH values for the four sorted cell populations (Table 2). Because these ΔCt values represented the normalized expression of two housekeeping genes against each other, they would be expected to be very similar across the sorted cell populations. This was actually the case for GAPDH and β actin (Table 2, compare ΔCt β actin-GAPDH values for the four sorted cell populations), and to a lesser extent also true for HPRT and 18S, where C cells differed from the other sorted cell populations (Table 2, compare ΔCt HPRT1-GAPDH and ΔCt 18S-GAPDH values for the four sorted populations). These results validated GAPDH as well as β actin for normalization of RT-PCR data (Table 2, compare ΔCt β actin-GAPDH values for the four sorted cell populations), in full agreement with the Western blot results, where equal expression of GAPDH and β actin protein was observed across sorted populations (Fig. 2). Furthermore, the RT-PCR results showed that our protocol was applicable to both low (HPRT) and high (18S) abundance mRNA targets (Table 2).

To further explore the biological validity of the RT-PCR results, we calculated the ΔCt calcitonin-GAPDH values, representing normalized expression of the calcitonin mRNA. Calcitonin gene expression is expected to be very high in sorted C cells, virtually absent in sorted follicular cells, and intermediate in thyroid cells. Exactly, this expression pattern was in fact observed (Table 2, compare

ΔCt calcitonin-GAPDH values across the four sorted populations. Low ΔCt values correspond to high calcitonin mRNA expression). While we did not compare the efficiency of the calcitonin and GAPDH RT-PCRs, the ΔCt values were in complete agreement with the Western blotting and IRMA data (Figs. 2B and 2C). Thus, our flow sorting protocol was able to capture expected cell type-specific gene expression profiles (Table 2).

Finally, to explore the robustness of our protocol, we compared RT-PCR data from two independent sorting experiments (Table 2). On average, ΔCt values differed by 2.2 between experiments (Table 2). Additionally, in cases where larger discrepancies were observed between the experiments, this affected low-abundance as well as high-abundance targets (Table 2, ΔCt HPRT-GAPDH entry for C cells, and ΔCt Calcitonin–GAPDH entry for Non C-/follicular cells). Thus, the sorting protocol appeared to provide reproducible quantitative RT-PCR data.

In summary, our laboratory is interested in developing generally applicable methods for sorting rare cell subsets from organ material based on intracellular markers, for molecular toxicology studies. To our knowledge, very few studies have described sorting of permeabilized cells for molecular studies (1–3,14,15). Importantly, most of the published studies have utilized cell cultures (as opposed to organ material), or utilized DNA staining only (as opposed to immunostaining for intracellular antigen) (1,2,14,15), or performed PCR (as opposed to RT-PCR) (16). Barrett and coworkers sorted epithelial cells from mammary biopsy specimens based on cytokeratin staining, but did not attempt to sort rare cell subsets, and only RT-PCR analysis was performed on the sorted cells (3). In contrast, we validated our sorting protocol on a rare cell population, and showed that it is compatible with peptide, protein as well as low abundance mRNA analysis. In this regard, it should be mentioned that the methanol fixation used in our protocol is known to be compatible with phosphoprotein analysis (5), an important feature, given the importance of protein phosphorylation in regulating cellular functions in organs undergoing physiological or pathological changes.

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