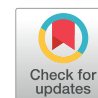

Single Molecule–Based fliFISH Validates Radial and Heterogeneous Gene Expression Patterns in Pancreatic Islet β-Cells

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ISLET STUDIES

Single-cell RNA-sequencing (scRNA-Seq) technologies have greatly enhanced our understanding of islet cell transcriptomes and have revealed the existence of β-cell heterogeneity. However, comparison of scRNA-Seq data sets from different groups have highlighted inconsistencies in gene expression patterns, primarily due to variable detection of lower abundance transcripts. Furthermore, such analyses are unable to uncover the spatial organization of heterogeneous gene expression. In this study, we used fluctuation localization imaging–based fluorescence in situ hybridization (fliFISH) to quantify transcripts in single cells in mouse pancreatic islet sections. We compared the expression patterns of Insulin 2 (*Ins2*) with *Mafa* and *Ucn3*, two genes expressed in β-cells as they mature, as well as *Rgs4*, a factor with variably reported expression in the islet. This approach accurately quantified transcripts across a wide range of expression levels, from single copies to >100 copies/cell in one islet. Importantly, fliFISH allowed evaluation of transcript heterogeneity in the spatial context of an intact islet. These studies confirm the existence of a high degree of heterogeneous gene expression levels within the islet and highlight relative and radial expression patterns that likely reflect distinct β-cell maturation states along the radial axis of the islet.

In recent years, single-cell RNA-sequencing (scRNA-Seq) technologies have facilitated the identification of cell-type-specific transcriptome signatures of the individual islet cell populations and enhanced our understanding of β-cell heterogeneity with respect to their maturation and functional state, as well as overall health (1–6). However, due to the difficulties associated with detecting lower abundance transcripts, comparison between scRNA-Seq data sets from different groups has shown variable results for genes reported to being heterogeneously expressed in β-cells (7,8). Comparing between scRNA-Seq data and data acquired from sorted β-cell populations has shown clear inconsistencies, with the identification of only few markers of heterogeneity. With the exception of a small number of abundant gene transcripts, most gene transcripts were detected in only a subset of β-cells, masking true cell heterogeneity as a result of working near the detection limit (7,8). These limitations emphasize the need to validate scRNA-Seq data using other single-cell analysis approaches, such as quantitative mRNA fluorescence in situ hybridization (FISH) or immunofluorescence. Single molecule–based FISH approaches can provide quantitative analysis of transcript counts without the biases imposed by cell dissociation and destruction or cDNA amplification and do not require the availability of antibodies. These approaches also provide the spatial information needed to determine whether cell subpopulations, such as cells at different maturation or endoplasmic reticulum stress levels (1), and heterogeneous gene expression are spatially driven, as previously demonstrated (9,10).

In this study, we used a high-accuracy single molecule–based FISH approach, fluctuation localization imaging–based FISH (fliFISH) (9,11), to quantify the expression of selected genes in single cells in pancreatic islet cryosections

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to better evaluate their heterogeneity in the spatial context of the islet. We focus on three genes that are expressed at different levels in subsets of β-cells and in non–β-cell islet populations in comparison with Insulin 2 (*Ins2*) expression. v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (*MafA*) and Urocortin 3 (*Ucn3*) are both believed to be expressed in β-cells as they mature (12–14). Similar to most transcription factor genes, *MafA* is expressed at relatively low levels. Regulator of G protein signaling 4 (*Rgs4*) has been functionally analyzed in the developing pancreas and in β-cells (15,16), yet a recent single-cell study identified it as primarily an α-cell marker (2). fliFISH analysis clearly demonstrates that there is a wide range of transcript copies for each of these genes in β-cells. For all four genes, we found that cells with highest transcript copies are located near the center of the islet, while cells with low copies are located along the periphery. This radial pattern is especially obvious for *Ins2*, consistent with the idea that more mature β-cells are found near the center of the islet, while β-cells at the periphery are likely to be at an earlier maturation stage (17). *Rgs4* transcripts are found in nearly all islet cells, with a similar radial expression pattern as *Ins2*. The radial expression pattern is less obvious for *Mafa* and *Ucn3*, which tend to have a narrower range of transcript numbers in adult β-cells. However, similar relative expression levels are often found for all four genes, in which a single cell expresses either higher or lower levels of all four transcripts. The similar relative and radial expression patterns, together with the heterogeneous expression levels, likely reflect distinct β-cell maturation states along the radial axis of the islet.

RESEARCH DESIGN AND METHODS

Mouse Pancreatic Tissue Sections

Animal studies were approved by the Institutional Animal Care and Use Committee at University of Colorado. Pancreata were isolated from 8-week-old wild-type C57BL/6J mice, washed in PBS, fixed in 4% paraformaldehyde/PBS overnight, and then transferred to 30% sucrose/PBS overnight at 4°C. Samples were embedded in OCT and sectioned at 8–10-μm thickness. To identify the islets, the sections were subjected to immunostaining using primary antibody against insulin (#3014; Cell Signaling Technology), followed with a secondary antibody tagged with Alexa 800, as previously described (9).

fliFISH Probe Design and Analysis

fliFISH was used following the approach describe earlier (9,11). Each primary FISH probe contained a sequence of ∼20 oligonucleotides complementary to target mRNAs, followed by a sequence of 28 oligonucleotides overhang, complementary to secondary probes. Eight probes were designed to target each mRNA. Secondary probes were tagged with two photo-switching dye molecules of the same color (Atto 488, TMR, Alexa 594, or Alexa 647), one at each end. All probe sequences are listed in Supplementary Table 1. Hybridization followed the protocol described in detail earlier (9,11). fliFISH analysis was done as described earlier (9,11), with the following exception. After finding the centroid for each photo-switching fluorescent spot showing on-off emission or blinking pattern (18), a blinking density map was generated in which the value of each pixel indicates the number of blinking events within distance R of that pixel. Such map was used to group blinking events into clusters (or transcripts). R was determined by the length of the target mRNAs and the probe localization error upon hybridization, which in our study was determined to be ∼30 nm. To evaluate the transcript numbers per cell, the cell boundaries were outlined, guided by the insulin immunofluorescence signal, and the nuclei stained with DAPI. The analysis was performed using MATLAB routines available upon request.

Fluorescence Microscopy

Imaging was performed using an Olympus IX-71 inverted microscope with a 100 times oil immersion objective, five solid-state lasers (405, 488, 542, 594, and 640 nm), and an Electron Multiplying CCD camera (iXon Ultra 897; Andor). The camera's pixel size was 16 μm, and the microscope magnification was ×100. Thus, the scale factor was 160 nm/pixel. A total of 10,000 images of 512 × 512 pixels were collected within 400 s and stored as 16-bit FITS files. Imaging was done in oxygen-depleting buffer (9).

Data and Resource Availability

The data sets generated and analyzed during the current study are available from the corresponding authors upon reasonable request.

RESULTS AND DISCUSSION

In this study, we used fliFISH to quantify gene expression in islet cells in mouse pancreatic tissue sections. fliFISH is built on principles used in single molecule–based super-resolution fluorescence imaging, such as stochastic optical reconstruction microscopy or photo-activation localization microscopy, in which photo-switching fluorophores are used. By tagging the FISH probes with photo-switching fluorescent dyes, fliFISH enables the detection and counting of individual transcripts with high resolution and accuracy (9). Figure 1 shows an example of an islet in which the transcripts for *Ins2*, *MafA*, *Ucn3*, and *Rgs4* are presented as dots in green, red, blue, and magenta, respectively. The islets were identified by immunofluorescence targeting insulin (grayscale), and the nuclei were stained using DAPI, together helping to distinguish between neighboring cells and guiding the segmentation of the cells (dashed lines in Fig. 1).

Transcript counts for *Ins2*, *MafA*, *Ucn3*, and *Rgs4*, as quantified by fliFISH, are shown in Fig. 2 for each islet β-cell. Counts for each gene are indicated by the number shown in each cell, as well as by the intensity or shade of the respective colors. The color intensity was assigned by

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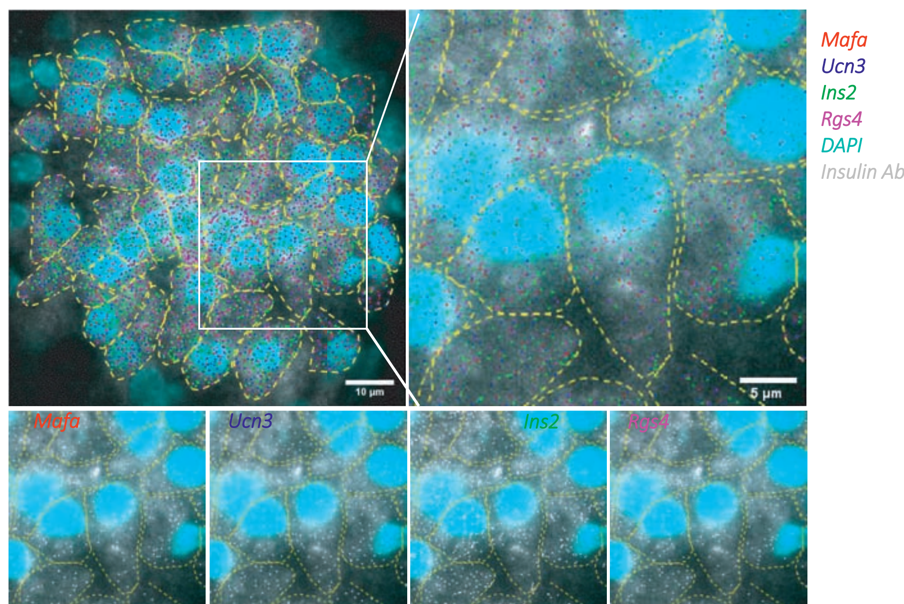

Figure 1—An example for a fliFISH image of an islet (left), identified in the cryosection by immunoassay using an antibody (Ab) against insulin (grayscale). Individual cells within the islets were outlined (yellow dashed lines), guided by the immunoassay pattern and the DAPI-stained nuclei (light blue). Transcripts for *Mafa*, *Ucn3*, *Ins2*, and *Rgs4* are shown as red, blue, green, and magenta dots, respectively. The enlarged area marked by the square is shown on the right. The four images in the bottom panel show the transcripts as white dots for each gene individually.

the ratios between the transcript count in the cell and the highest count found in that islet per cell. In other words, color intensity reflects normalized transcript levels. Thus, cells with relatively high transcript counts are shown in intense or bright colors, while cells with relatively low transcript counts are shown in faint or light colors. Single-cell gene expression mapping of additional islets can be found in Supplementary Fig. 1. To evaluate the specificity of our approach, we compared the counts of *Ucn3* transcripts per cell in pancreatic islets from *Ucn3* knockout and wild-type mice. Supplementary Figure 2 presents representative islets, showing low levels of background counts in the knockout islets (likely due to low levels of nonspecific binding and autofluorescence), which are significantly lower than the counts in the wild-type islets. If these low counts reflect residual *Ucn3* expression, the slightly lower counts in cells at the islet edge could potentially result from their proximity to the acinar RNAses. However, Supplementary Fig. 4 shows that cells with very high GAPDH counts or even the highest counts can appear at the islet edge, ruling out the involvement of acinar RNAses.

The maps of transcript counts for *Ins2*, *MafA*, *Ucn3*, and *Rgs4* demonstrate the large variability in the expression levels of the four genes across the islet cells. Transcript copies variability can span nearly two orders of magnitude, from only 1 copy to >100 copies/cell (Supplementary Fig. 3), even in the same islet (Fig. 2 and Supplementary Fig. 1). Although in most cells, the number of *Ins2* transcripts is higher than the numbers of the other three genes, the difference is smaller than the difference observed in RNA-Seq data. This discrepancy might reflect a limitation of single molecule–based FISH techniques, in which the counts of highly expressed transcripts can be underestimated since tightly clustered transcripts can potentially be counted as one transcript.

Cells with the highest copy numbers are often found near the center of the islet, while cells with low copy numbers are found along the edge of the islet. The radial pattern is especially obvious in the expression of *Ins2*, in which cells around the edge can express only a couple of transcripts, while cells in the center can reach >170 transcripts. This radial pattern is consistent with the notion that β-cells near the center of the islet are more mature than those at the periphery (10,17). Although to a lesser extent, the radial pattern also shows in the expression of *Rgs4*, in which cells at the edge contain only a few transcripts and cells at the center express >70 transcripts. While nonspecific binding (Supplementary Fig. 2) could potentially decrease the difference in the counts of cells in the center versus the edge of the islet, they cannot eliminate the radial pattern, especially not for *Ins2*, in which the count difference in cells at the islet edge versus the core can reach two orders of magnitude.

The radial pattern is less obvious in the expression of *MafA* and *Ucn3*, perhaps due to their overall reduced heterogeneity in expression, although the cells with the least number of *MafA* or *Ucn3* transcripts were generally located at the periphery. Interestingly, similar relative expression levels are often found for all four genes in a

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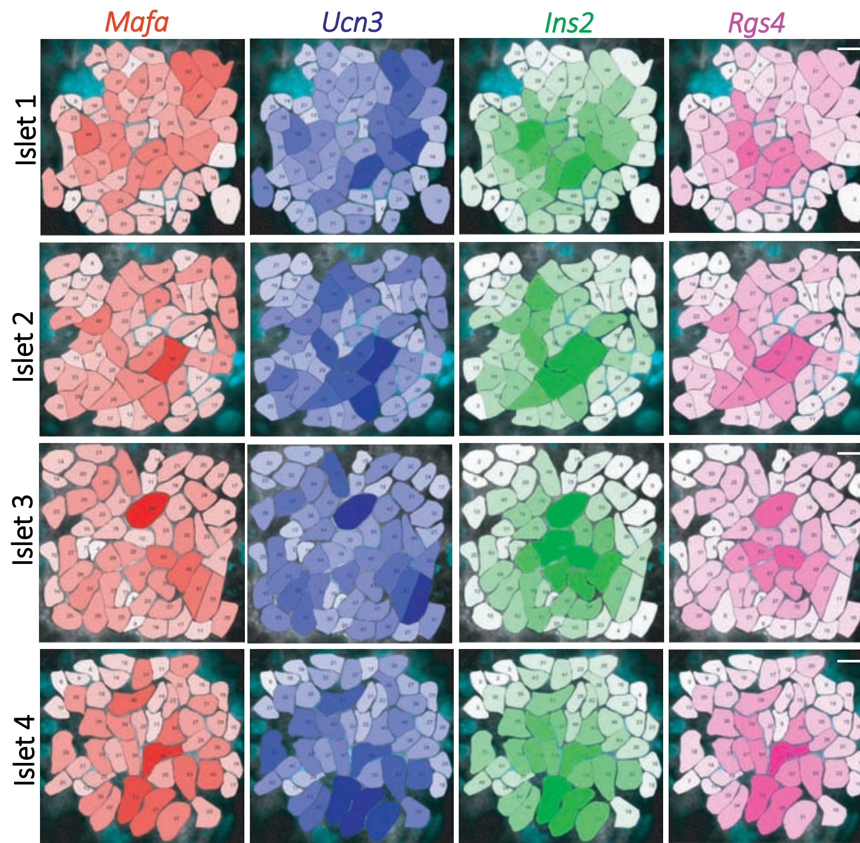

Figure 2—Mapping gene expression levels in single islet cells. Each horizonal panel shows the same islet, in which transcript counts for *Mafa* (red), *Ucn3* (blue), *Ins2* (green), and *Rgs4* (magenta) are indicated by the numbers placed over each cell. Each cell was assigned an intensity level or shade of the respective gene colors based on the ratios between the transcript counts in the cell and the highest count found in that islet per cell. Thus, cells with high transcript counts are shown in intense or bright colors, while cells with low transcript counts are shown in faint or light colors. Scale bars = 10 μm. Mapping of additional islets is shown in Supplementary Fig. 1.

given cell, with the same cells expressing higher levels and other cells expressing lower levels for all four genes (19). The similar relative expression levels found in a given cell may reflect a greater extent of cellular maturity and/or functionality, as demonstrated in a recent β-cell study using a novel Patch-sequencing technique to correlate β-cell exocytosis and gene expression (15,16). As a control experiment, the copy number of GAPDH, a housekeeping gene expected to be expressed in all cells, has been quantified. Supplementary Figure 4 presents representative islets, showing relatively uniform expression levels in all cells with no radial patterns across the islets. These results also indicate no increase in transcript degradation in cells along the edge of the islets.

Although *Rgs4* has been shown to be expressed in β-cells and is a regulator of β-cell function, a recent scRNA-Seq profiling the transcriptome of islet cell populations identified it as an α-cell–enriched gene (2). The presence of *Rgs4* transcripts in α cells is also confirmed in this study by fliFISH (Supplementary Fig. 5). Our observations confirm the presence of *Rgs4* transcripts in β-cells; however, many of the β-cells within the islet express relatively low *Rgs4* transcripts, which may be below the level of detection for most scRNA-Seq platforms.

To evaluate the ratios between the relative expression levels of *Ins2*, *MafA*, and *Ucn3* per cell in the spatial context of the islet, we assigned colors to cells using a combination of green, red, and blue shades, respectively (Fig. 3). As in Fig. 2, normalization to the highest transcript count found in that islet per cell was first applied for each gene to assign shade values for each cell in each of the three colors (red [*Mafa*], blue [*Ucn3*], and green [*Ins2*]). The shade values were then combined for each cell to generate a mixed red, green, and blue color per cell. Further, weighted transparency was applied to all cells according to the ratio between the total transcripts in the cell and the highest total transcript found in that islet per cell. Thus, brighter cells have higher total transcript counts than darker cells. The mixed colors assigned to each cell reflect ratios between normalized transcript levels, in which, for example, brighter green cells indicate relatively high levels of *Ins2* transcripts and relatively low levels of *Mafa* and *Ucn3* transcripts. Likewise, cells in bright magenta express roughly equal copies of *Mafa* and *Ucn3*,

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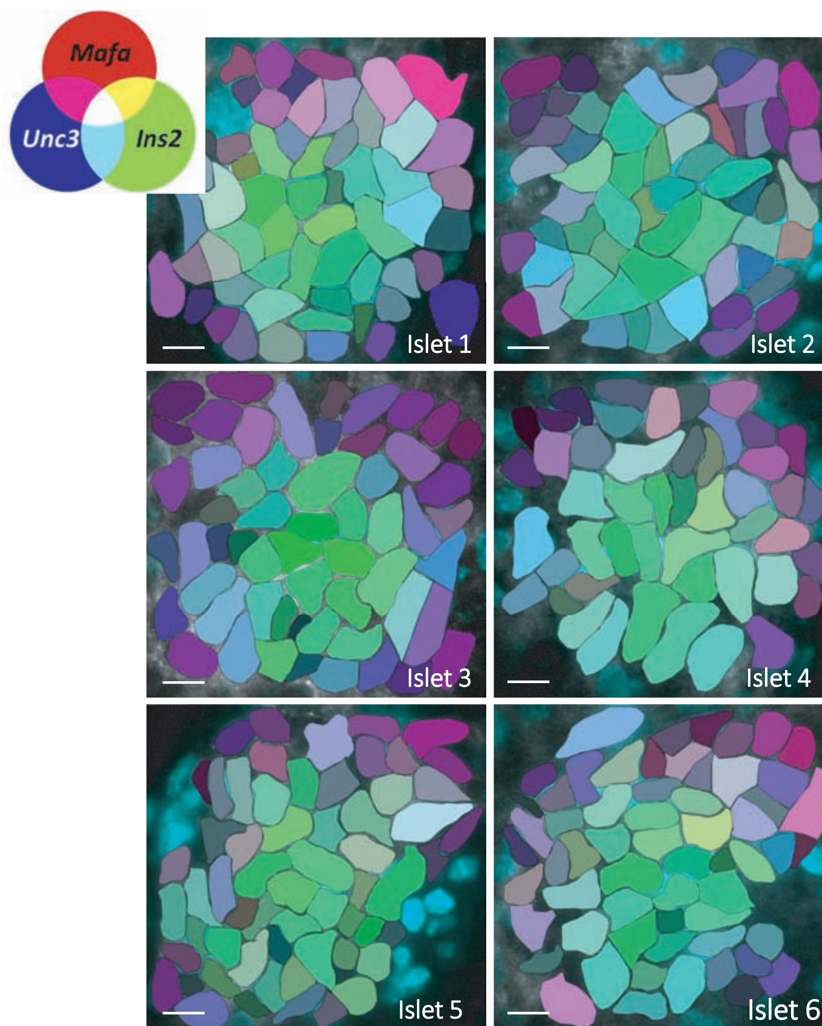

Figure 3—Mapping ratios among relative expression levels of *Mafa*, *Ins2*, and *Ucn3* in individual islet cells. The ratios among *Mafa*, *Ins2*, and *Ucn3* relative transcript levels was mapped using combinations of red, green, and blue (Venn diagram in top left corner), respectively. The relative values for each gene per cell were determined by calculating the ratio between each gene transcript count and the highest count of the gene found in that islet per cell. Further, weighted transparency was applied according to the ratio between the total transcripts in the cell and the highest total transcript found in that islet per cell. Thus, cells in brighter green shades, for example, express relatively higher numbers of *Ins2* transcripts compared with the relative expression levels of *Mafa* and *Ucn3* transcripts, while cells in brighter red or blue shades express relatively higher numbers of *Mafa* or *Ucn3*, respectively. Likewise, cells in bright magenta express roughly equal copies of *Mafa* and *Ucn3*, both at relatively high levels, with no or relatively very low levels of *Ins2* copies (see Venn diagram). Scale bars = 10 μm. The islets are the same as those shown in Fig. 2 and Supplementary Fig. 1 according to their numbers.

both at relatively high levels, with no or relatively very low levels of *Ins2* copies.

The ratio mapping in Fig. 3 confirms that cells in the center of the islets tend to have relatively higher *Ins2* counts (brighter green shades) compared with cells closer to the periphery. The low expressing cells at the periphery might represent β-cells at an earlier maturation stage, possibly originating from the "neogenic niche" at the edge of the islet, as described by van der Meulen et al. (10). Cells at the far edge of the islets, which show very low expression levels for all three genes, may represent the immature β-cell population described in van der Meulen et al. (10) to have no or low levels of *Ucn3* and *MafA*. Interestingly, fliFISH analysis shows that all β-cells expressing *Ins2* transcripts also express *MafA* transcripts, albeit often at very low levels. This observation is in contrast to published scRNA-Seq (8,20), which could only identify MafA transcripts in ~50% of the β-cells.

Our study demonstrates the power of fliFISH to identify and validate the existence of β-cell heterogeneity, in which single-cell expression levels can span over nearly two orders of magnitude. Our study also shows clear relationships between the heterogeneity and the spatial organization of the islet, in which radial gene expression pattern is observed, with low copy numbers at the periphery and high copy numbers at the center of the islet.

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Author Contributions. F.L. processed the samples, acquired and analyzed the data, generated the figures, and helped write the manuscript. D.H. helped with microscopy setup and analysis programs. C.D. dissected the tissue and prepared the cryosections. C.A. contributed to discussion and reviewed the manuscript. L.S. and G.O. wrote the manuscript. G.O. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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