APPLICATION NOTE

High Throughput, Parallel Quantification and Imaging of Sperm Thioredoxin SPTRX-3 in Defective Human Spermatozoa by ImageStream Flow Cytometer: Combining Sperm Morphology with Flow Cytometry

**Clayton Buckman,^{1,2*} Thaddeus C. George,⁶ Sherree Friend,⁶ Miriam Sutovsky,^{1,2}
Antonio Miranda-Vizueté,⁵ Christophe Ozanon,^{4*} Phil Morrissey,⁶ Peter Sutovsky^{1,2}**

¹Division of Animal Sciences, ²Departments of Obstetrics, Gynecology & Women's
Health, ³Cell and Immunology Core, School of Medicine, University of Missouri,
Columbia, MO 65211-5300, USA

⁴FERTILITY, Assisted Fertilization Medical Center, UNILAB Laboratory, Clinique
Monplaisir, 69008 Lyon, FRANCE

⁵Centro Andaluz de Biología del Desarrollo (CABD-CSIC), Dpto. de Fisiología,
Anatomía y Biología Celular, Universidad Pablo de Olavide, 41013 Sevilla, SPAIN

⁶AMNIS Corp., 2505 3rd Ave, Suite 210
Seattle, WA 98121, USA

Running Title: Concomitant flow cytometry and imaging of defective spermatozoa

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ABSTRACT

Spermatid specific thioredoxin-3 protein (**SPTRX-3**) accumulates in the superfluous cytoplasm of defective human spermatozoa. Novel ImageStream technology combining flow cytometry with high throughput cell imaging was used for parallel quantification and visualization of SPTRX-3 protein in defective spermatozoa of infertile men. The SPTRX-3 containing cells were overwhelmingly spermatozoa with a variety of morphological defects. Image Stream quantification of SPTRX-3 surpasses the informative value of both conventional flow cytometry and light microscopic semen evaluation. The observed patterns of the retention of SPTRX-3 in the sperm samples from infertility patients support the application of SPTRX3 as a biomarker of male infertility.

RESULTS & DISCUSSION

Flow cytometry and epifluorescence microscopy combined with biomarkers of sperm quality/fertility are useful techniques in andrology (Cordelli et al., 2005), but neither method allows to directly correlate the quantity of said biomarkers with sperm morphology. ImageStream technology (Amnis Corp., Seattle, WA)(George et al., 2004) combines the high throughput of flow cytometry (up to 100 cells/second) with single cell imaging microscopy allowing the capture of up to 6 images of each passing cell (bright field, side scatter and up to 4 channels of fluorescence imagery). A set of five semen samples from consenting infertility patients were analyzed by ImageStream flow cytometer (**Figure 1**) after labeling with fluorescently conjugated antibodies against

spermatid specific thioredoxin 3 (SPTRX-3), a male germ-line specific protein uniquely retained in the superfluous cytoplasm of defective human spermatozoa (Jimenez et al., 2004). Sperm samples stained with anti-SPTRX-3 antibody followed by TRITC conjugated secondary antibody (Goat-anti-mouse IgG-TRITC) were shipped to Amnis Corp. headquarters and supplemented with blue fluorescent DNA dye DAPI at 5 μ g/ml (Invitrogen-Molecular Probes). Samples were run on the ImageStream with both 405 nm and 488 nm laser excitation. Approximately 10,000 cells were collected per sample and the images obtained were corrected for spectral crosstalk using single color controls. Cell imagery was collected in the absence of bright field illumination for the purposes of constructing a compensation matrix. The four-panel imagery shown in **Figure 2** includes bright field, DAPI, SPTRX-3, and a composite of the bright field, DAPI and SPTRX-3 images. In order to identify single cells from debris and clumped cells, a bivariate dot plot of aspect ratio (minor axis over major axis) and intensity of the nuclear stain was created (**Figure 2 A**). The major population of single cells was found and gated as R1. The veracity of the gate was checked by examining the imagery within the gate and along the threshold of the gate. The SPTRX-3 positive (R3) and negative (R2) single cells were then gated on the SPTRX-3 intensity histogram (**Figure 1B**). Brightfield, DAPI, SPTRX-3, and Composite images of representative cells from each population are shown in **Figure 1C and D**.

Occasionally, an argument arises in flow cytometric sperm analyses regarding whether the high readings of sperm quality biomarkers are contributed by the actual spermatozoa or by residual bodies and cellular debris abundant in human semen samples (Marchiani et al., 2007; Muratori et al., 2007; Muratori et al., 2005). ImageStream

technology uniquely combines high throughput of flow cytometry with the ability of a digital microscopes to record images of individual measured cells passing through the flow cytometer. Consequently, we were able to directly examine cells within high and low-fluorescent populations gated off on the flow cytometric histograms generated by ImageStream (**Figure 2**). ImageStream analysis revealed the patterns of SPTRX-3 retention in the defective sperm subpopulation. Such an analysis proved unambiguously that the flow cytometric “events” responsible for high SPTRX-3 signal indeed correspond to spermatozoa with superfluous cytoplasm around the midpiece or inside the nuclear vacuoles.

As an alternative to conventional sperm morphology analysis, the SPTRX-3-based assay and other biomarker-based sperm assays could be employed as the reference laboratory tests using ImageStream technology. To our knowledge, this is the first published result of ImageStream application in andrology. In the past, we had to use flow cytometric sperm sorting to visualize the defective spermatozoa labeled by antibodies recognizing the biomarkers of male infertility. Such laboriously sorted samples were then screened by epifluorescence microscopy, and the information was extrapolated between flow cytometry and microscopy. Combining the high throughput of flow cytometry with the resolution and informative value of epifluorescence microscopy, ImageStream analysis would allow an andrologist to immediately identify the prevailing types of defects and determine the relative abundance of SPTRX-3-positive spermatozoa. In a clinical setting, such information could be easy to pass on to a patient, and could even be used to monitor changes in sperm quality that could occur in the course of a couple’s treatment following lifestyle advice.

FIGURE LEGEND

Figure 1: ImageStream 100 instrument manufactured by AMNIS Corporation, Seattle, WA.

Figure 2: Typical data outputs of ImageStream flow cytometer, combining flow cytometric scatter diagrams (**A**) and histograms of SPTRX-3-induced fluorescence intensity (**B**) with bright field imagery (**C, D**; column BF), and with epifluorescence imaging of sperm DNA (column DAPI) and SPTRX-3 (column SPTRX-3). Brightfield/DAPI/SPTRX3 composite images of individual spermatozoa acquired with the ImageStream flow cytometer are also shown (**C, D**). During ImageStream data acquisition, single cells (**A**, gate R1) are first separated from debris or multicellular events (**A**; dots outside of gate R1). The SPTRX-3 fluorescence intensities of the single cells within gate R1 are plotted on a histogram (**B**), and the low fluorescent, SPTRX-3-negative (R2), and highly-fluorescent, SPTRX-3-positive (R3) populations are gated in. Panel **C** shows examples of SPTRX-3-negative spermatozoa as viewed directly in population R2. Panel **D** shows SPTRX-3-positive spermatozoa (as well as one residual body) as viewed directly in subpopulation R3. Note that most spermatozoa in panel **D** show SPTRX-3 labeling around the sperm tail midpiece and connecting piece.

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