Spotlight

Microtechnology & Nanotechnology

MicroRNA Profiling of Individual Hematopoietic Stem Cells

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Single cell studies are increasingly important to stem cell biology as rare cell subsets are found to play a critical role in the maintenance of processes such as hematopoiesis. MicroRNAs are regulated during hematopoiesis, and the ability to study the expression levels of these genes in single cells is crucial to understanding the role microRNAs may play in hematopoietic stem cell function. Purified hematopoietic stem cell populations are heterogeneous, making it challenging to study these rare cells when data are averaged over an entire population. This report presents the use of microfluidics to assemble and conduct thousands of small-volume microRNA expression assays, using individual cells from different hematopoietic populations, including highly purified stem cells. Multiple miRNAs are assayed from single cell lysates using quantitative real-time PCR, and the resulting data have the accuracy and precision required to detect biological variability in expression levels among individual cells.

Introduction

Understanding the function of individual cells demands new experimental approaches capable of analysing minute amounts of material. An ideal model for single cell applications is hematopoiesis, the continual process of blood cell production via differentiation of hematopoietic stem cells (HSCs) into mature blood cell lineages *(Figure 1)*. HSCs represent a small fraction of the total cells present in bone marrow and in the circulating blood, and so cannot be studied in tissue samples or large pools of cells. Fluorescence activated cell sorting (FACS) can be used to select various hematopoietic cell types based on expression of cell surface markers. However, known stem cell markers yield populations of limited purity, so even carefully sorted populations remain heterogeneous. Averaging data from these populations results in the substantial loss of information specific to stem cells, since signal from minority stem cells is swamped by the contributions of more abundant progenitor cell types. Furthermore, stem cell populations may contain sub-populations with subtle phenotypic variations that can only be detected with single cell assays.

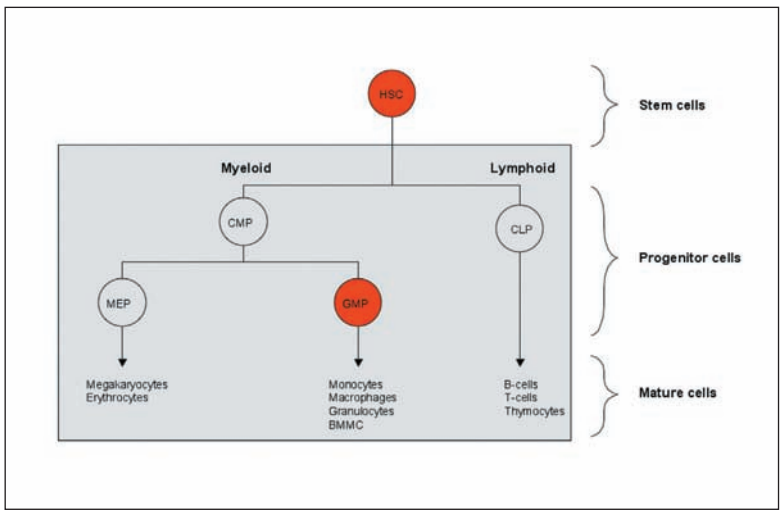

Figure 1. Hematopoietic cell lineages. Hematopoietic stem cells (HSCs) are self-renewing, and also give rise to progenitor cells that differentiate into the various blood cell types. Single cells were drawn for miRNA expression experiments from three populations. Two of these populations, HSCs and granulocyte-monocyte progenitor cells (GMPs), are shaded in red. The third population consists of bone marrow progenitor and mature cells (cell types within grey box).

MicroRNAs (miRNAs), which regulate expression of genes during development and other biological processes, have been found to be important players in hematopoiesis [1]. The expression of miRNAs differs widely between different tissues and cell types, emphasising the degree to which miRNA expression itself is controlled [2]. Accurately determining expression of miRNA genes in hematopoietic stem cells requires a single cell approach capable of high throughput. A large number of cells must be examined to determine, statistically, whether miRNA expression levels are similar in many cells, or to identify sub-populations within a FACS-sorted population of cells. Additionally, it is preferable to assay multiple genes per cell, to obtain a more complete and useful profile of each cell.

Microfluidics provides the ability to perform established techniques such as real-time PCR on the miniaturised scale required when working with the limited sample obtained from single cells. In the precisely controlled volumes of microfluidic circuits, reactions on multiple targets can be automatically carried out in nanolitre volumes, maintaining high concentrations of target molecules to improve reaction efficiency while minimising the use of reagents [3]. Additionally, because the architecture of the chip tightly controls reaction volumes, assays are highly reproducible with the accuracy and precision needed to detect small differences in expression.

Advances in microfluidics have also increased the throughput of real-time PCR. With the 96.96 Dynamic Array Integrated Fluidic Circuit (IFC) from Fluidigm, for example, 9216 distinct PCR reactions can be assembled and run on a single chip with a fraction of the pipetting steps otherwise required.

This paper reports the feasibility of assaying the expression of multiple miRNA genes from single cells. With the high throughput provided by IFCs, standard curves and multiple controls for each assay can be simultaneously assayed in a single experiment for high quality quantitative data. In a recent report, this approach has been applied to different populations of single hematopoietic cells, and reactions were found to be sufficiently precise to detect biological variation among individual stem cells [4].

Methods

Bone marrow cells were extracted from C57/B6 mice *(Figure 2)* and sorted by fluorescence activated cell sorting into hematopoietic populations based on cell surface markers as described in the literature [4]. Single cells from hematopoietic stem cell ($CD45^+/CD48^-/EPCR^+/CD150^+$), granulocyte-monocyte progenitor (GMP) cell ($lin-/ckit^+/sca\text{-}1^-/CD34^+/Fc\gamma R^{high}$) and bone marrow cell ($CD45^+/CD48^+$) populations were manually picked using pulled glass capillaries, and released into 4.35µl reverse transcriptase (RT) reaction mix: 1x RT Buffer from High Capacity cDNA Reverse Transcription Kit (ABI), 12.5 nM pooled stem-loop RT primers (12-plex, ABI), 1 U/µl RNase inhibitor, 0.1% Tween, 0.2 pg/µl Cel-2 template as spike-in control).

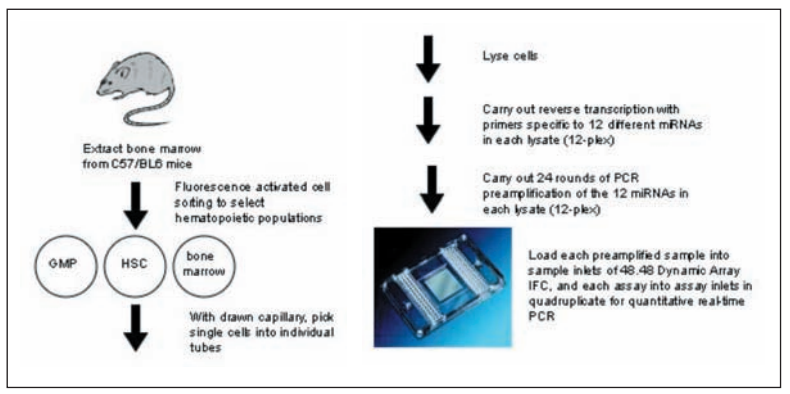

Figure 2. Experimental procedure. Bone marrow was collected from C57/BL6 mice, and hematopoietic cell populations sorted based on cell surface markers. Single cell studies were conducted on three populations. Reverse transcription and preamplification were carried out as 12-plex reactions, with the primers for 12 different miRNAs at once. The preamplified samples were combined with the TaqMan assay for each gene on the microfluidic chip.

Negative no template controls consisted of 2 tubes with 1µl PBS added to the RT reaction mix, and 2 tubes with 1µl of cell supernatant for each cell population.

To determine the reproducibility of each real-time PCR assay on the chip, 10 single cells were picked into 43.5µl. After lysis, 9 single-cell equivalents from this pool were aliquoted in 4.35µl each, enzyme mix added and RT reaction carried out as described below.

To generate a standard curve against which the individual cell results could be measured, a sample containing 10^6 hematopoietic cells/ml was lysed, and a dilution series consisting of 1000, 100, 10, 1, and 0.1 cell equivalents was created in triplicate. Enzyme mix was added and RT reaction carried out as described below.

Cells were lysed by heating to 95°C for 5 minutes in RT mix and 0.65µl enzyme master mix was added to each tube to obtain final concentrations of 5 mM dNTPs, 3.35 U/µl MMLV reverse transcriptase, and 0.26 U/µl RNase inhibitor.

A pulsed RT protocol was used to perform RT reaction: 16°C for 30 minutes, followed by 60 cycles of 20°C for 30 seconds, 42°C for 30 seconds, and 50°C for 1 second. A final 5 minutes incubation at 85°C inactivated RT enzyme.

A preamplification master mix was assembled, and 20µl was added to each tube to obtain final concentrations of 1X TaqMan® Universal Master Mix No AmpErase® UNG mix, 0.02X pooled (12-plex) TaqMan assays (ABI), 4 mM MgCl2, 0.8% Tween, 4 mM dNTPs, 0.25 U/µl AmpliTaq® Gold. Preamplification was carried out with the following conditions: 95°C for 10 minutes, followed by 24 cycles of 95°C for 15 seconds and 60°C for 4 minutes.

Following preamplification, the reactions were diluted to 100µl with 75µl of H_20. 1.67µl of diluted reaction was added to 5µl of sample loading mix (3.7µl 2X Universal Master Mix (ABI), 0.37µl Fluidigm sample loading reagent, 0.93µl TE buffer). TaqMan assays were prepared by diluting 1:1 with the assay loading reagent. 6µl of each sample were loaded to the sample inlets, and 6µl of each of the 12 assays were loaded in quadruplicate to the assay inlets of two 48.48 Dynamic Array IFCs. The samples and assays were automatically combined on-chip *(Figure 3)* using the BioMark™ system; this achieved a total of 4608 real-time PCR reactions, with cycle threshold (Ct) values calculated and plotted as a heat map.

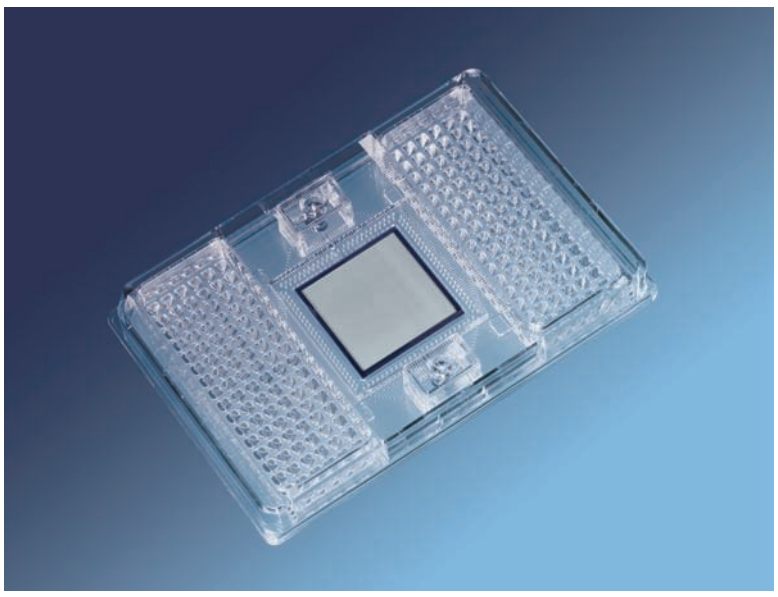

Figure 3. Matrix PCR reaction assembly. On the 48.48 Dynamic Array IFC, samples and PCR primer-probe sets are loaded into the sample and assay inlets respectively, and combined within the architecture of the chip into 2304 unique PCR reactions.

Results

Experiments were designed such that reverse transcription (RT) and preamplification were carried out as 12-plex reactions. Control experiments have determined 12x to be the optimal multiplex level to reliably detect abundant miRNA from single cells [4].

Initial experiments were conducted to confirm that less than one cell equivalent was sufficient to quantitatively assay miRNA levels. Pools of cells were lysed, and diluted such that 1000, 100, 10, 1 and 0.1 cell equivalents entered the 12-plex RT step. For each miRNA assay, the miRNA input vs. Ct value was linear over the dilution curve including 1 cell equivalent *(Figure 4)*. Assays for which single cell Ct values fell outside of the linear range or were smaller than the no template control were excluded from the analysis.

Nine technical replicates containing the equivalent amount of miRNA from a single-cell were assayed to examine the reproducibility of the quantitative PCR reactions. As shown in *Figure 5*, very high precision was observed, with replicate assays differing by 2-fold or less from the average. Technical variation was less than one Ct value for single cells [4], indicating that the assays are capable of detecting a 2-fold difference in initial target for reactions with 100% efficiency.

Single cells were then assayed, 20 single cells from a homogeneous population (Granulocyte-monocyte progenitor cells (GMP)), 20 single cells from a highly heterogeneous (bone marrow) population, and 20 single cells from a highly purified hematopoietic stem cell population (HSC) *(Figure 1)*. The reactions were run in quadruplicate, and the resulting Ct values were plotted as a heat map *(Figure 6)*.

The Ct values for the cells from the homogeneous population are very similar from cell to cell, while the Ct values for the cells from the heterogeneous population demonstrate a range in miRNA levels, which confirms the ability to detect biological variations such as those observed in the HSC population. In each case, the four replicate measurements for each cell show high precision.

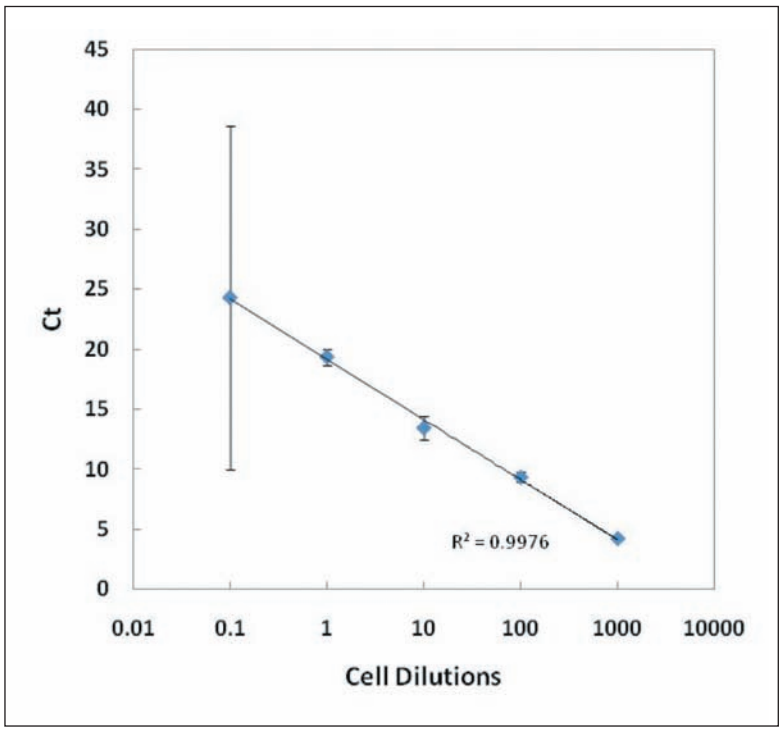

Figure 4. Ability to quantify micro RNAs from less than one cell equivalent of total miRNA from hematopoietic cells. After multiplex reverse transcription and multiplex preamplification, the quantitative PCR assay for miR-223 produces a linear response between input RNA and Ct values even below 1 cell equivalent of miRNA.

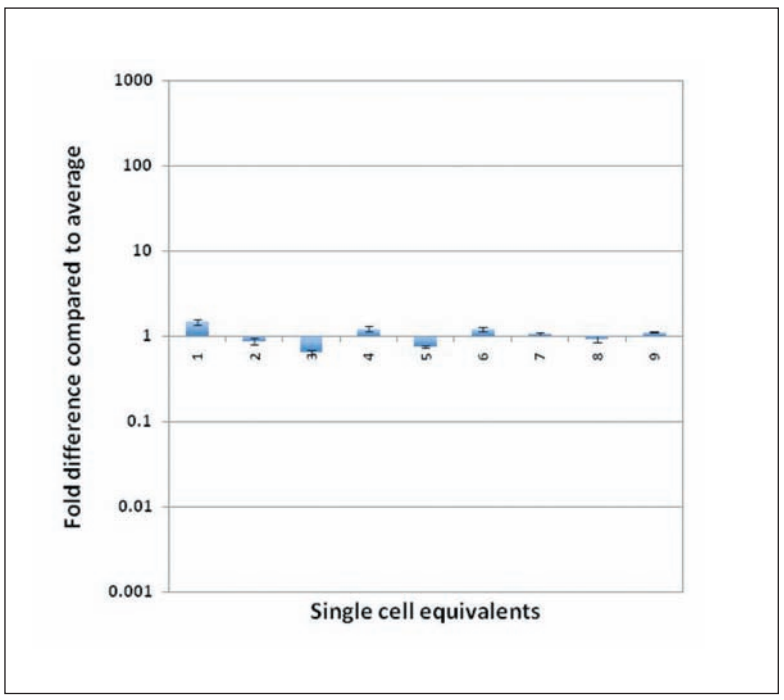

Figure 5. High technical precision when assaying single cell equivalents of miRNA. The quantitative PCR assay for miR-223 was carried out on nine aliquots of pooled HSC lysate, each equivalent to the miRNA found in a single cell. The assay is seen to be highly reproducible when single cell equivalents are assayed.

Discussion

Hematopoietic stem cells are essential for renewal of blood cell populations. Understanding the biology of these cells is relevant for clinical applications including the treatment of cancer and blood-borne diseases. HSCs are relatively rare, comprising only 1 of every 100,000 hematopoietic cells, so must be separated from the bulk of hematopoietic cells to be studied. Even after FACS sorting, stem cell populations are not homogeneous; different stem cells will give rise to different frequencies of differentiated lineages. Therefore, single cell studies have the potential to provide insight into HSCs, perhaps leading to the identification of new markers that can be used to identify different subpopulations within the sorted HSC population.

Since miRNAs are implicated in playing a role in regulation of hematopoiesis [1], examining the miRNA expression profile in HSCs provides valuable information. Recently, the first high-resolution mapping of miRNA expression in hematopoietic cell populations was published [4]. This work involved high-throughput quantitative PCR to complete expression analysis of 288 miRNAs in over 20 different hematopoietic cell types. The miRNA expression levels were found to cluster with cell type, supporting the contention that miRNAs help regulate differentiation of these populations. It was also observed that individual stem cells exhibited greater variability than individual cells of homogenous populations, such as GMP *(Figure 1)*.

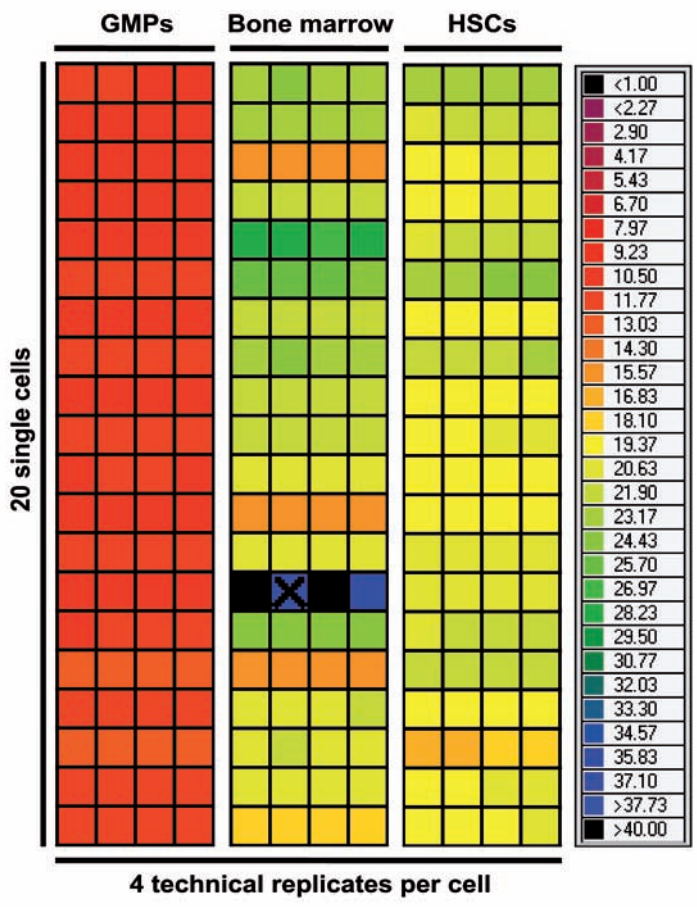

Figure 6. Biological variation detected in individual cells. The micro RNA miR-223 was quantified in 20 single cells from a homogeneous cell population (GMP), a heterogeneous cell population (bone marrow) comprising progenitor and mature cells, and a highly purified HSC population. Four replicates were conducted on each cell, and the Ct values for each assay are shown on a heat map. The Ct values for the GMP cells are very similar, indicating that expression levels of this gene are comparable in this homogeneous cell type. In contrast, expression of miR-223 varies greatly among the heterogeneous cells, reflected in the different Ct values observed among the bone marrow cells. Biological variations can be observed in the highly purified HSC population.

The work presented here demonstrates the ability to conduct real-time quantitative PCR reactions to assay miRNA expression levels in single cells, with sensitivity *(Figure 4)* and precision *(Figure 5)* necessary to detect biological variation *(Figure 6)*. Conditions for multiplex reverse transcription and preamplification have been developed [4] to increase the molar concentration of targets and the number of miRNA genes that can be assayed from a single cell in nanoliter volumes.

New techniques to study gene expression in single cells are needed, since traditional high-throughput expression studies using microarrays require microgram amounts of total RNA, equivalent to the amount that can be obtained from tens of thousands of cells. Linear amplification can increase nucleotide amounts by 1000 fold while maintaining the initial relative representations of each species [5], reducing the amount of starting material needed, but still requiring a large number of cells.

Quantitative real-time PCR more accurately quantifies RNA levels, and can be conducted with as little as one cell's worth of RNA. However, with traditional technologies, qPCR has not been able to match the throughput of microarrays. Even with robotic assistance, PCR carried out in 384 well plates requires dozens of plates, dozens of 3-hour PCR protocols, and thousands of pipetting steps to match a typical array.

The microfluidic approach presented here increases the throughput of quantitative real time PCR, making it possible to accurately assay expression of many genes from many single cells at once. The optimisation of multiplex reverse transcription and preamplification from single cells increases the amount of information obtained from each cell. Multiplexing can impact the efficiency of quantitative PCR, so the ability to concurrently run a serial dilution standard curve alongside single cell assays allows the data to be calibrated and fold-change values to be accurately calculated. The high-throughput nature of microfluidic chips allows numerous controls and standard curves to be assayed simultaneously with the single cells, for high-quality and quantitative data.

The nanolitre reaction volumes of the Dynamic Array microfluidic chips reduce reagent consumption by 100- to 200-fold over larger plate-based PCR reactions, resulting in significant savings. Also, since reaction volumes are determined by the architecture of the chip, volumes are tightly controlled, leading to high reproducibility between reactions. The reproducibility extends even to single cell reactions *(Figure 5)*.

It has been shown that IFCs make possible precise and quantitative miRNA gene expression analysis from large numbers of single cells.

Acknowlegements

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Cell Receptor Recycling Mechanism Discovery Opens up New Class of Therapeutic Targets

Professor Manojkumar Puthenveedu of Carnegie Mellon University studies the mechanisms by which membrane trafficking controls and co-ordinates the complex signalling pathways in the brain. Despite the fact that almost all diseases can be traced to a defect in how cells respond to signals, little is known about how signalling pathways are maintained in normal cells and the changes that occur in abnormal conditions. Using the **Andor** Revolution XD Confocal Microscope for live cell studies, Professor Puthenveedu has led an international team of researchers to elucidate how signalling receptors are recycled to the cell membrane. The discovery of the mechanism by which signalling receptors travel back to the surface of the cell after activation and internalisation opens up a new class of therapeutic targets.

The team used live cell confocal fluorescence microscopy to label and image beta-2 adrenergic receptor (b2AR), the receptor for adrenaline and noradrenaline and one of a group of G protein-coupled receptors (GPCRs) pivotal to the regulation of heart and lung function, mood, cognition and memory, digestion, and the inflammatory response. After internalisation, they found that b2AR was recycled via unique domains on the endosome, which they termed Actin-Stabilized Sequence-dependent Recycling Tubule (ASSERT) domains. Rapid turnover of endosomal actin was confirmed by FRAP (fluorescence recovery after photobleaching). The ASSERT domains trap and slow down the release of receptors to provide a slower pathway compared to the faster bulk recycling pathway and could potentially be targeted by pharmaceutical agents to control diseases resulting from abnormal cell signalling.

"Confocal fluorescence microscopy has been the driver for an explosion in the study of dynamic processes in living cells, but not all systems are created equal. We have created an instrument that optimises sensitivity, acquisition speed and specimen viability," said Mark Browne, Director of Systems at Andor. "The high optical efficiency of the Revolution XD spinning disk system allows the use of reduced laser power and, therefore, lowers the risk of phototoxicity to the specimen during long term experiments while Andor's IQ software ensures precise synchronisation of illumination, detection and scanning for unparalleled image quality and acquisition rates."