Final Report

OTKA K 82097

Individual variability in the stress response of genetically homogeneous mammalian cell population

Heat shock response is one of the most ancient and evolutionarily conserved protective mechanisms found in nature. The induction of heat shock proteins (HSPs) is an important unifying component of most of these responses, and this induction has proven to be essential for survival under stressful conditions. The study of the cellular stress response is of great importance to our understanding of how cells respond and adapt to various changes in their environments especially during different pathophysiological conditions. Regulation of HSP expression is intricate, with multiple layers of redundancy and feedback control, but a small family of transcription factors called heat shock factors (HSFs) are the primary regulators of stress-inducible expression in eukaryotic cells. According to our present knowledge hsp genes can be regulated by protein denaturation, membrane fluidity or microdomain organization, RNA structure and redox control. In the past two decades, a membrane sensor model has been developed in our laboratory which predicts the existence of a membrane-associated stress sensing and signaling mechanism from prokaryotes to mammalian cells [Vigh et al., 2007a,b; Török et al., 2003; Nagy E et al., 2007]. According to this model changes in the physical state or composition of lipid molecular species with the concomitant reorganization of membrane microdomains (rafts) may serve as the molecular switch for the operation of these "cellular thermometers". Knowledge on gene expression and cellular responses in cells is derived from analyses of populations consisting of millions of genetically identical cells. These isogenic populations exhibit broad phenotypic variation driven by cell cycle, cell ageing and epigenetic regulation. Fundamental questions relate to whether simultaneously cultured highly diverse cells suffer the same consequence of an imposed stress and whether they respond in the same manner. The present knowledge of gene expression and cellular responses is known to derive from analyses of heterogeneous populations. Although this approach provides useful insights into average population responses, they do not furnish information on individual cells or subpopulations. In the present project we aimed to characterize the individual variability in the stress response of genetically homogeneous mammalian cell population by combining classical flow cytometry with the state of the art ultrasensitive high content imaging techniques. Our specific objectives were 1) to link the population heterogeneity of the heat shock response and membrane structure (raft organization and dynamics) in mammalian cell cultures 2) to characterize lipidomics and proteomics of sub-populations responding to identical stressor at different levels 3) to elucidate the importance of cell cycle at the level of individual cells. The identification of specific changes in membrane domain structure leading to selective refinement of heat shock proteins in a heterogeneous cell population could help us to understand why a small subpopulation of cells could determine the outcome of important disease states.

RESULTS

Method development

To accomplish our objectives we retailored our molecular imaging laboratory in order to be able to follow when and where genetically or biochemically defined molecules, signals or processes appear, interact and disappear, in time and space. With high-content cellular analysis, it is necessary to perform high-throughput phenotype profiling, linking gene expression to biochemical signaling pathways in the cell and, ultimately, to cell behavior. During or after the imposition of stress on cells we zoom in on individual cells or individual fluorescently tagged molecules, using ultrasensitive, high-content, time-lapse fluorescent microscopy to observe what happens as each cell reacts to the particular treatment. A change may occur in the topology of a particular membrane domain, a candidate from which an initial heat shock signal could originate, or a membrane receptor may be activated, allowing the monitoring in real time as a receptor complex responds and activates signaling pathways. The particularly valuable aspect of this methodology is not the astounding visual images it produces, but rather the abundant and diverse data that can be extracted from those images—data that afford a better understanding of what is happening in the cell in response to stress. Image analysis of this nature requires both state of the art instrumentation and image processing algorithms to correct for optical aberrations, efficiently segment objects (cells, cellular compartments, signaling clusters, membrane domains etc.). In the current project we established robust tools for automatic image analysis to follow the individuality of dynamic cellular events (from seconds to ms timescale) in a population of cells during the development of the stress response. To gain insight into the correlation between membrane organization and the HSR the software were optimized to follow membrane dynamics at a very high spatial and temporal resolution in time lapse microscopy on large number of cells.

Instrumentation

During the first year of the project we were focusing on the development of new tools required to carry out the proposed research. We installed a new, fast camera into our ultrasensitive fluorescence microscope setup with which it became possible to follow the dynamics of membrane domains and fluorescence molecules within them on the millisecond timescale thereby significantly increasing the spatial temporal resolution of our system.

We also introduced a second light source, a fluorescent lamp monitor the fluorescence signal reporting the stress response (fluorescent proteins driven by heat shock promoter) while measuring membrane dynamics with our original laser (TIR) illumination (fluorescent membrane probes) at the same time. We have developed new acquiring software with the help of an undergraduate information technology student who participated on a student contest (TDK) with his innovative “Ultrasensitive, fast, high resolution microscopic imaging system control” project (Vigh, 2012). With the new controlling software it became possible for us to follow the events described above on hundreds of cells within a short time interval (<1 min) thereby to profile the stress response in time on a large population of cells at the individual cell level. Since the processes we aimed to monitor are extremely temperature sensitive we have also developed a new control system to adjust sample

temperature with high accuracy on the sample stage in the microscope thereby we are able to image cells under physiological or if needed under stress conditions for days.

Image processsing

Cell membranes are higly dynamic, consisting of multitude of interacting subdomains. The length scales of these membrane associations span a wide range of magnitudes ranging from small nanometer sized cholesterol rich rafts to large micron-sized platforms. These highly heterogeneous structures exhibit dynamics on the millisecond timescale. Hence, the dynamic organization of the cell membrane can only be understood by the application of techniques that incorporate spatial as well as temporal measurements of diffusion, and thus provide a picture of how the membrane works as a system on a larger scale. Most of the available imaging techniques for mapping membrane dynamics are not fast enough for automatic image acquisition on large number of cells thereby we introduced a new technology in collaboration with the National University of Singapore (Sankaran et al., 2009). This technique is Image FCS which is a temporal fluorescence correlation spectroscopy by the use of fast ultrasensitive imaging wich makes it possible to map dynamical parameters very quickly (acquition time ~10s) on hundreds of pixels at the same time. Spatiotemporal image correlation is achieved in total internal reflection (TIR) mode using the newly installed ProEM EMCCD. The time resolution used in the TIR mode is about 1ms. We established a semiautomatic data acquisition for the characterization of preselected cells by measuring membrane dynamic maps on each of the cells as a funcion of time.

Population heterogeneity of the heat-shock response

We applied time-lapse microscopy on heat-shocked CHO, B16 mouse melanoma and SHSY5Y human neuroblastoma cells expressing hsp70 promoter-GFP reporter construct to measure the expression levels of the fluorescent reporter in hundreds of cells at the same time for 24 hours by using fast scanning of large areas of cells (0.5 cm2) (Fig 1B). Following heat shock all cell lines showed a pronounced heterogeneity in their response to the stress. To correlate membrane dynamical and structural events with the observed heterogeneity we imaged the membranes as well.

Population heterogeneity of the membrane structure

Membrane mapping of heat shocked cells was carried out with GPI-GFP constructs stably expressed in CHO cells or with fluorescent probes added externally (f-PEG-Chol, Bodipy SM) to cells. Following the segmentiation of the high resolution images we determinded the average size of the labeled membrane domains and the total membrane area covered by them for each cells on a large imaged area containing hundreds of cells. Similarly to the stress response the membrane structure also showed heterogeneity both in the size and in the total area of domains on the membrane surface. The cells were classified based on their responsiveness to heat shock (Fig 1A).

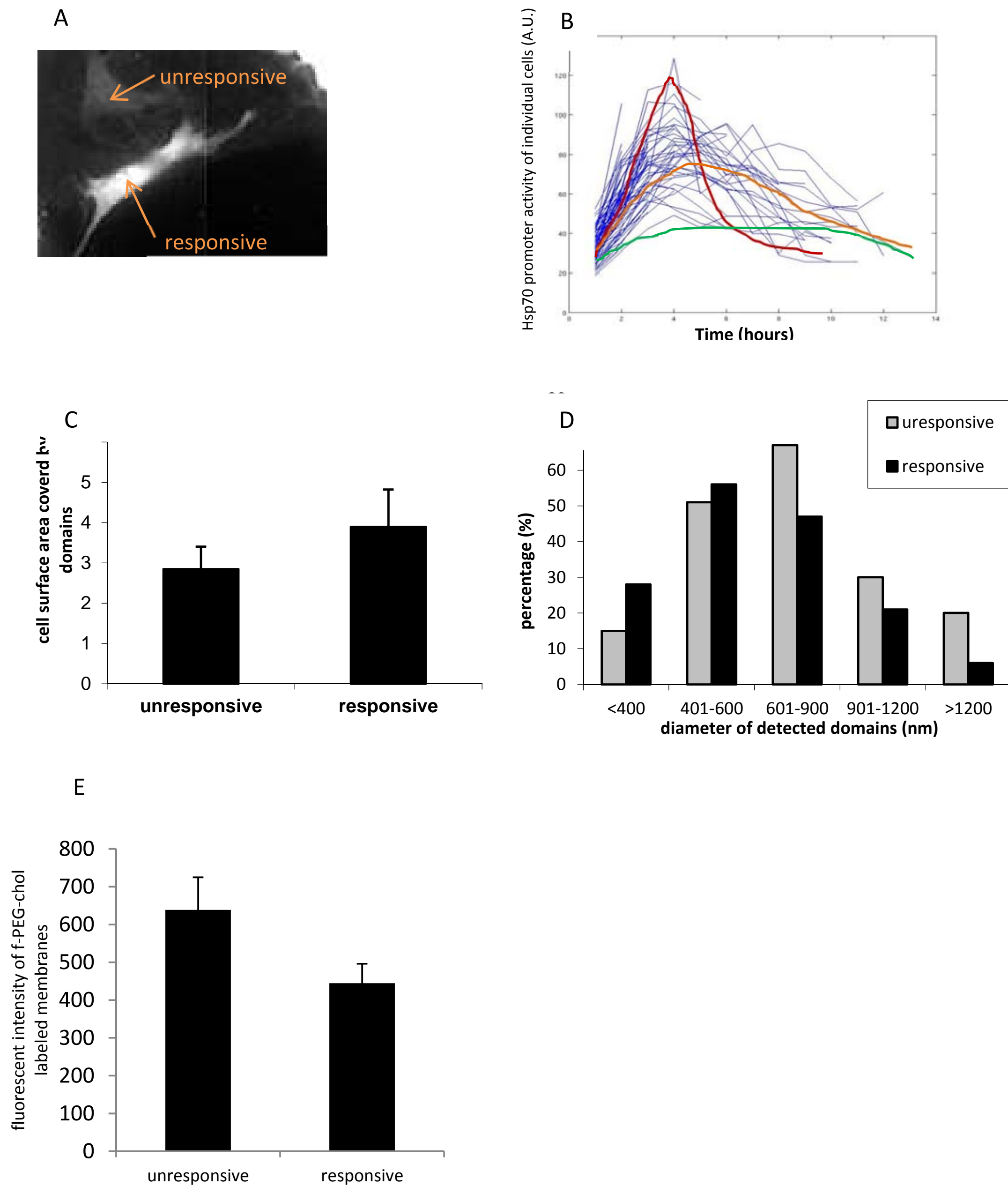


Fig 1. Heat shock profiling of fluorescein-PEG-Cholesterol (f-PEG-chol) labeled B16-F10 mouse melanoma cells expressing Hsp70 promoter driven YFP.

A) Responsive and unresponsive cells. B16 cells were heat shocked for 1h at 42°C followed by 6h recovery at 37°C. YFP fluorescence was measured in a Zeiss Axiovert200 microscope using the cell profiling setup. B) Dynamics of Hsp70 promoter activity of individual cells (red, orange and green lines show typical types of responses). C) Correlation between responsiveness and the cell surface area covered by f-PEG-chol labeled domains. D) Size distribution of detected domains. D) Size distribution of detected domains. E) Correlation between responsiveness and the fluorescence intensity of f-PEG-cholesterol labeled domains (which correlates whit the cholesterol content of the membrane)

The total membrane area covered by the f-PEG-cholesterol labeled cholesterol rich raft domains was significantly larger in case of the responsive cells (Fig 1.C). The average domain size in the responsive cells however was detected to be half of the size measured in case of the responsive cells (Fig 1D). Most interestingly, responsive cells appeared to have a lower cholesterol content as it could be estimated by the integrated intensity of f-PEG Chol signal (Fig 1E) as described (Sato et al, 2004; Peter et al, 2013). Unlike filipin and other cholesterol probes, this molecule could be applied as an aqueous dispersion, and when added to live cells, fPEG-Chol distribute exclusively in the outer plasma membrane leaflet and is enriched in microdomains. Thereby cholesterol content seems to play a crucial role in the perception and/or signalling for response. The large, high-cholesterol containing rafts may interfere with the stress signalling. These observations are in agreement with our previous data demonstrating that activation of the HSR using mild thermal stress coincides with Chol-rich membrane raft reorganization (Nagy et al. 2007). The 'quality' of pre-existing microdomains may determine the extent of the HSR. We observed that the changes in the expressions of hsp25 and hsp70 were paralleled by modulation in the Chol content of microscopic Chol-rich domains in a cell number-dependent manner (Figures 7 and 8 in Péter et al., 2012). It is noted that BGP-15, a known chaperon co-inducer (Crul et al, 2013) was able to permeate into the surface membranes of B16 cells and to associate prefentially to Chol-enriched lipid platforms thus preventing the transient structural disintegration of rafts induced by fever type HS. This compound was able to remodel Chol-enriched lipid platforms together with the restoration of diminished HSR in high cell number cultures (Gombos et al. 2011). To get further insight into the observed cholesterol dependent heterogeneous response apart from following the changes in the morphology and composition, we started the characterization of membrane dynamics as well by measuring the diffusion properties of fluorescent membrane probes localized in the different domains in the membrane by image FCS (Fig 2.) and single particle tracking (Gombos et al, manuscript in preparation).

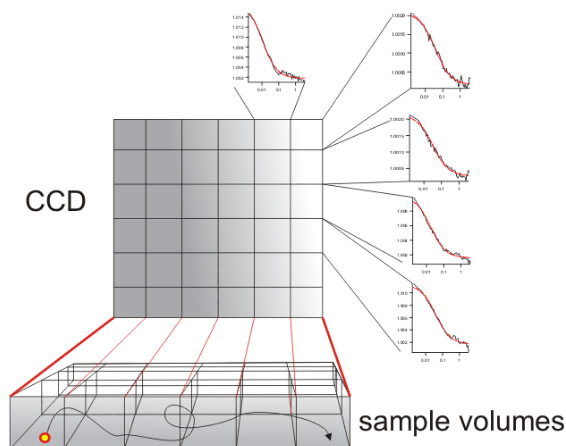


Fig 2. Schematic draw of image based FCS measurement. *Individual autocorrelation functions could be fitted for every pixels of the recorded movie. Pixels correspond to small sample volumes of the measured object.*

The distributions of the different lipid classes that make up cell membranes, including sphingolipids, cholesterol, and glycerophospholipids, are highly heterogeneous. The membrane exhibits a range of

diffusion coefficients due to the presence of regions of lower mobility (called ‘‘lipid rafts’’) that are embedded in a fluid phase of higher mobility. To investigate the cell membrane organization, we used CHO cells labeled with GPI-GFP construct (Fig 3). The diffusion coefficient can be used as a measure of the fluidity of the membrane. With this method we are capable of continuously monitoring the diffusion of selected markers and their interaction with the membrane ultra-structure.

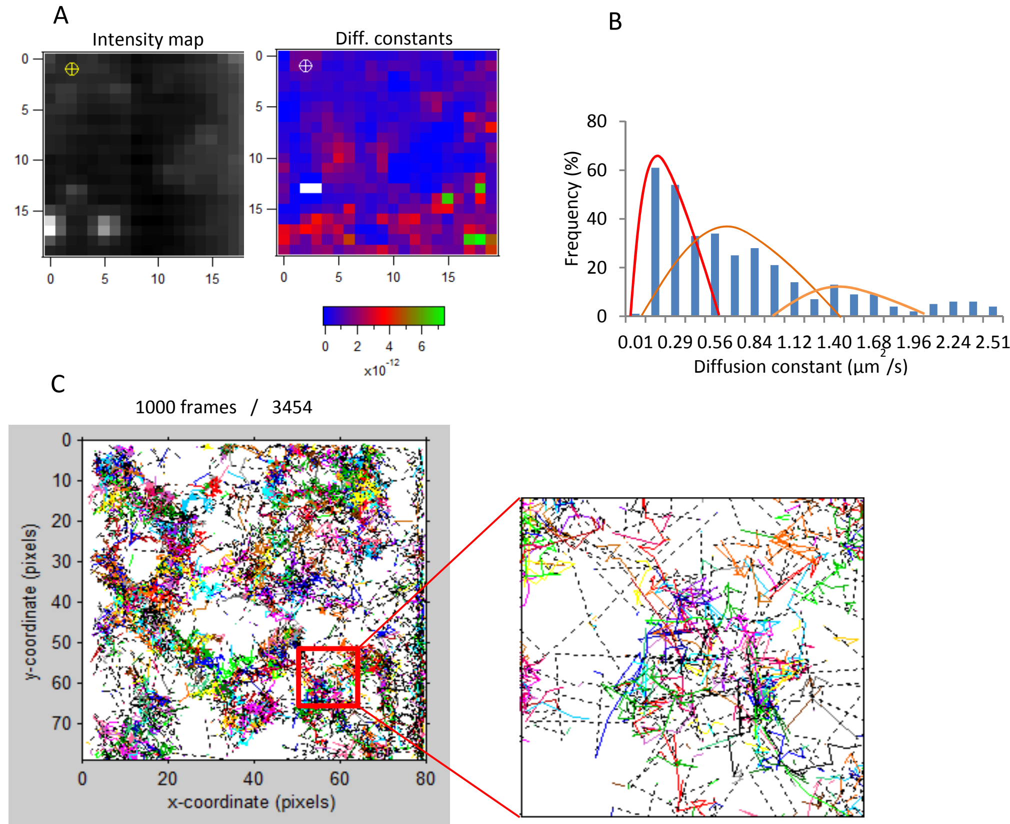


Fig3. Zooming into membrane dynamics with ultrasensitive fluorescence microscopy. *20000 frames long movies with 465 or 240 frame/sec frame rate were taken on mGFP expressing CHO cell membranes. A.) ImFCS analysis showing both the integrated fluorescence intensity map and an informative map of the diffusion constants of the given membrane area. On this map fields of slower (blue colored) and faster (red colored) molecular dynamics could be observed. B.) Histogram on distribution of the diffusion constants derived from the previous map. Different populations of the dynamics could be identified. Changing of this distribution can inform us about a membrane organization change. C.) Single molecule tracking analysis of 1000 frame/the same 20000 frames long movie. Colored lines indicates single trajectories of mGFP-GPI labeled rafts.*

This real-time capability permits us to study changes in structural domain associations as a consequence of membrane perturbations such as temperature and we can dissect the series of events occuring as a result of the signalling process. We observed a limited diffusion of the raft associated GPI anchored GFP and revealed that the preexisting quality of the diffusion-limited

domains would determine the effectivity of the cellular response. (Gombos et al., manuscript in preparation). By mapping the diffusion on the membrane we found a close correlation between membrane content, raft structure and in plane diffusion of membrane domains. Studies are under way to sort cells responding differently to perform in depth lipidomics to reveal other factors contributing to the structural plasticity found in cells responding more efficiently to stress.

Effect of cell-cycle

The heterogeneity of gene expression among individual cells may reflect physiological differences between cells like different stages of cell cycle or replication, or exposure to different local microenvironments. In the proposal we planned to establish a model for cell-cycle by using a YFP-tagged cell-cycle reporter YB1 (Y-box binding protein-1) which is especially sensitive for cell cycle phase [Cohen et al., 2009). It turned out that the selection marker introduced into this contsruct was a second, red fluorescence protein, which interfered with our hsp promoter probing. Therefore we decided to use fluorescence cell cycle indicators available for live cell labelling. We tested Vybrant® DyeCycle™ Ruby stain designed for labeling the nucleus of live cells but, we realized that all the stained and pre-heatshocked cells were killed following illumination. We also tested Draq5, and cellTracker Green and cellTracker Red for long term time-lapse microscopy with the same detrimental result. Following a long optimization period we decided to follow two paths: 1) to synchronize cells and 2) to use another fluorescent cell cycle reporter construct the FUCCI system (fluorescent ubiquitination-based cell cycle indicator).

Our results show a very pronounced effect of cell cycle on the ability of cells to respond to stress. Synchronization experiments using serum starvation to trap cells to 90% in the G1 phase (Fig.3) showed that cells in this phase produce a very low HSP70 promoter activation (not shown). By using promoter probing and cell cycle indicators in parallel we observed that in an unsynchronized cell culture the majority of the cells in the S and G2 phases belong to the class of responsive cells, while cells in the G1 phase are less responsive or they respond at a later stage of the stress that is they may be more resistant to stress (Fig 4.). Our results are in agreement with previous observations for increased heat sensitivity of cells in S and G2/M phases (Rice et al., 1986). HSP70 was shown to be synthesized in a cell-cycle-dependent manner with highest concentrations within S phase [Milarski & Morimoto 1986; Hang et al., 1995, Hang et al., 1996, Zeise et al., 1998). De Laat and colleagues have demonstrated that the microviscosity, as measured by DPH-fluorescence polarization of synchronized neuroblastoma cells, changed markedly during the cell cycle, reaching a maximum in mitosis and a minimum during S phase (De Laat et al., 1980). Moreover, cell differentiation has also been associated with a progressive increase in microviscosity of the cell membrane (Shinitzky, 1984).

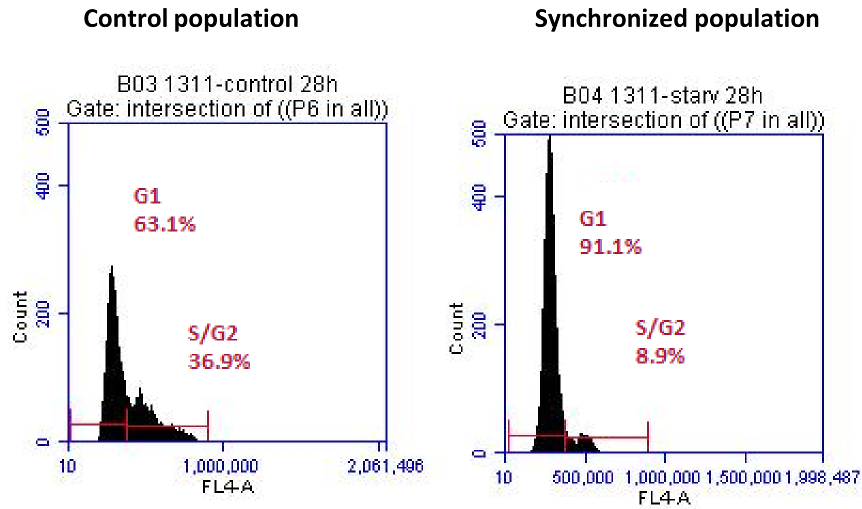
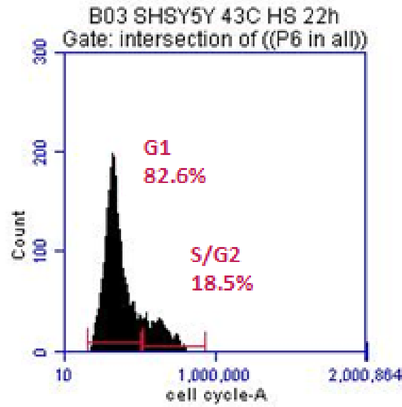


Fig3. Effect of serum starvation on cell cycle state of the population and heat shock response. Flow cytometric distribution of 28 hours serum starved SHSY5Y cells were obtained using DyeCycle™ for DNA staining. Ratios represents G1 and S/G2 phase

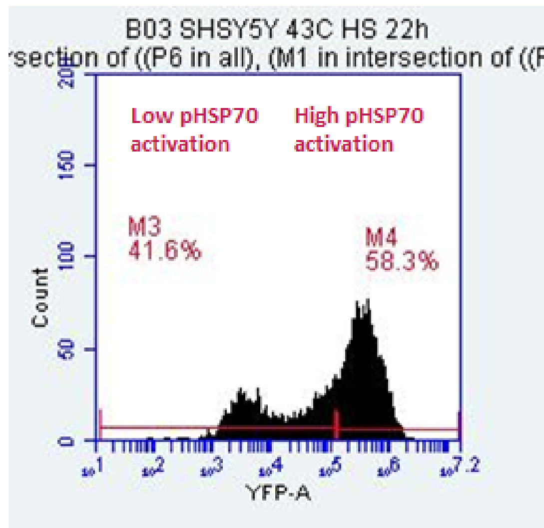
Interestingly, a recent study aiming to understand why replicating neuroblastoma cells in different cell cycle phases display different vulnerability to amyloid toxicity showed that membrane cholesterol (expressed as pg cell−1) was significantly higher in the G1 than in the G2/M and particularly in the S cell populations (Cecchi et al., 2008). An alteration of cholesterol homeostasis has also been proposed as a shared primary cause of several neurodegenerative diseases. Indeed, reduced levels of cholesterol have been found in brains from AD patients and loss of cholesterol in neuronal membranes enhances Aβ peptide production leading to neurodegeneration. Since heat shock proteins are playing an essential role in protein folding our findings connecting the dots between membrane composition, structure and HSR may have important projection on the field of disease states in which HSPs playing an active role.

The observed heterogenous HSR however cannot be explained solely with the cellular cell cycle progression since cells in the different phases are still not giving homogeneous response (Fig 4.). Detailed microscopic, proteomic and lipidomic studies are under way to characterize cell cycle dependent membrane reorganization in detail (Begüm Peksel, PhD work in progress).

Cell Population



G1 phase



S/G2 phase

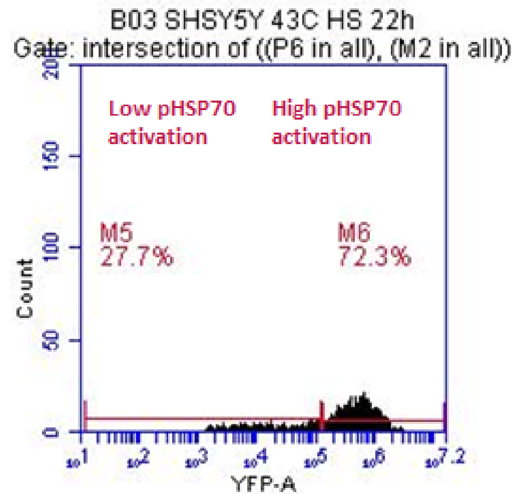


Fig4. Effect of cell cycle progression on the heat shock response. Flow cytometric distributions depicting hsp70 promoter driven YFP fluorescence from SHSY5Y cell populations gated by their cell cycle phase G1 and S/G2 determined with Dyecycle™

SUMMARY

In the present project we aimed to characterize the individual variability in the stress response of genetically homogeneous mammalian cell population and to identify membrane perturbations contributing to this phenomenon.

To accomplish these aims we set up a unique high-content ultrasensitive fluorescence imaging laboratory combining state of the art fluorescence correlation and single particle tracking with cell profiling technologies. We established robust cellular analysis tools to perform high-content

phenotype profiling, linking gene expression to biochemical signaling pathways and structural changes in the cell. We observed a heterogeneous response for all the tested cell lines. We found correlation between structure and composition of membrane domains and the ability and/or sensitivity of cells to respond to heat shock. We identified plasma membrane cholesterol content playing a crucial role in the perception and/or signaling for response. We showed that cell cycle, at least partially, is responsible for the observed heterogeneity but other factors should also be taken into account. Our experiments highlight the fact that caution should be taken during the study of heat shock response since the treatment itself could select for cells in the different cell cycle phase. The effect of mild heat treatment for instance could only be seen if the sensitive populations of cells in the S and G2/M phases are not lost during the stress.
Clinical conditions such as cancer, diabetes and neurodegenerative diseases are all coupled with specific changes in the physical state and lipid composition of cellular membranes and characterized by altered heat shock protein levels in cells suggesting that these "membrane defects" can cause suboptimal hsp-gene expression. Such observations provide a new rationale for the introduction of novel, heat shock protein modulating drug candidates. Intercalating compounds can be used to alter membrane properties and by doing so normalize dysregulated expression of heat shock proteins, resulting in a beneficial therapeutic effect for reversing the pathological impact of metabolic and neurodegenerative diseases (Crul et al., 2013).

Collaborations

In the course of the present project we established three new collaborations. 1) Together with Rachel Kraut and Kamila Oglecka from the Nanyang Technical University, Singapore we are investigating the use of ImFCS to study membrane domain structure and membrane trafficking, 2) with Thorsten Wohland from the National University of Singapore we are developing the novel use of imFCS technique, and 3) with Horváth Péter, ETH Zurich we are developing powerful image processing algorithms for microscopic stress profiling.

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