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9

Imaging Cellular Metabolism

Athanasios Bubulya and Paula A. Bubulya
Wright State University, Dayton, Ohio
USA

1. Introduction

Imaging tools that aid in identifying the precise location of diseased cells within a patient's tissues, and that measure the physiological status of these cells, have clear impact for medical scientists in a wide range of specialties ranging from clinical oncology to cardiology to neurology. Furthermore, laboratory scientists can utilize imaging methods to gain insight into the subcellular localization and kinetics of key branches for major biosynthetic pathways. Our predominant focus for discussion in this chapter is imaging of living cellular systems, and we also discuss how dynamics of key cellular pathways can be revealed from observations in fixed cells. We discuss some of the recent advances that continue to lead scientists toward imaging metabolic pathways for understanding and diagnosing human disease. The goal of this chapter is to provide the novice researcher with an overview of a variety of approaches for imaging cellular metabolism both for medical and research purposes.

Understanding the metabolic differences between normal cells and cancer cells is a major objective of biomedical research. Cancer cells exhibit increased metabolic rates for pathways needed to support uncontrolled cellular proliferation, and this has been exploited for treatment of tumors as well as for diagnostic purposes (Locasale et al., 2009). The problem for understanding and treating cancer is to learn not only what makes each specific type of cancer unique, but also to learn the commonly aberrant pathways in cancers such as higher glucose metabolism or altered membrane biosynthesis pathways that can be exploited as targets for developing diagnostic tools and anticancer therapies. There is continued hope for novel therapies not only in the well-documented oncogene/tumor suppressor-related cellular signaling pathways, but also in areas of renewed interest, such as unique regulation of stress response pathways by cancer cells (reviewed in Luo et al., 2009). Regardless of the pathway targeted, cancer therapy would ideally leave normal cells unaffected while specifically interfering with altered metabolic pathways of cancer cells.

2. Exploiting aberrant metabolism to image cancer cells

Increased glucose metabolism in cancer cells, initially observed by Warburg over 90 years ago, has fueled the development of labeled metabolites to differentially label cancer cells from surrounding normal tissues (Warburg, O., 1956). To detect the labeled malignant tissue, the use of minimally invasive in vivo imaging techniques has increased rapidly over the last two decades. Here we briefly describe various types of metabolites and the imaging

methods used to observe cancer cells in vivo. Of particular interest is the use of imaging techniques in conjunction with labeled metabolic markers to determine location, size and response to drug treatment of cancerous tissue in vivo. The current non-invasive imaging techniques include but are not limited to positron emission tomography (PET), magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT) and computed tomography (CT). These techniques have been extensively reviewed elsewhere and the reader is encouraged to refer to those recent articles (Plathow & Weber, 2008; Condeelis & Weissleder, 2010).

2.1 In vivo imaging probes

According to the Molecular Imaging and Contrast Database (MICAD), there are currently 1107 imaging probes and contrasting agents currently used for in vivo studies (Chopra et al., 2011). The most commonly used metabolic marker is the synthetic glucose analog, 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG) (Ido et al., 1978). FDG has wide clinical use because malignant cells display high glucose metabolic rates as compared to normal tissue (Warburg, 1956, Sokoloff et al. 1977). In the cell, FDG becomes phosphorylated by hexokinase, it can not be further metabolized, and it is in essence trapped within the cell. FDG uptake is therefore indicative of the rates of both glucose uptake and glucose phosphorylation. FDG uptake is monitored using PET and this method of probe detection has become the choice of clinicians for identifying tumors in vivo and has been used to successfully detect head and neck, prostate, breast, lung and liver cancer (Ben-Haim & Ell, 2009). Additional metabolic processes have been targeted for clinical use, and PET metabolic markers have been developed that detect apoptosis, angiogenesis, hypoxia, cell proliferation and amino acid metabolism. Metabolic markers currently being used include [^{11}C]Methionine, L-[3-^{18}F]-□-methyltyrosine [^{18}F]FMT, [^{11}C]Thymidine, ^{18}F-3'-fluoro-3'-deoxy-thymidine [^{18}F]FLT, [^{11}C]Choline [^{18}F]Choline, [^{11}C]Acetate, ^{68}Ga-NOTA-RGD [^{18}F]Galacto-RGD, ^{18}F-fluoromisonidazole [^{18}F]FMISO, [^{18}F]FAZA, ^{64}Cu-ATSM, ^{99m}Tc-Annexin-V and [^{124}I]Annexin-V (reviewed in Lee, 2010). Here we highlight PET markers that are associated with cell proliferation, amino acid metabolism and lipid metabolism.

Collectively [^{11}C]Methionine, [^{18}F]FMT, [^{11}C]Thymidine and [^{18}F]FLT are used as indicators of cell proliferation. These compounds can further be classified into amino acid and nucleic acid analogs. The radiolabeled amino acid [^{11}C]methionine, is easily taken up by tumor cells due to their increased protein synthesis, and has been very useful for identifying malignancies in the central nervous system (Comar et al., 1976, reviewed in Nanni et al., 2010). Furthermore, the amount of [^{11}C]methionine present in malignant tissue can be used to determine tumor grade (reviewed in Nanni et al., 2010). For example, [^{11}C] methionine, was recently used to grade the aggressiveness of glioblastoma in patients with grade IV gliomas (Kawai et al., 2011). Additionally, [^{11}C]Methionine/ PET is a promising tool for determining active tumor regions providing valuable information for chemotherapy (Tsien et al., 2011). Deng et al. (2011) recently synthesized S-^{11}C-methyl-L-cysteine as a PET tracer and suggest that it shows improved distinction between malignant tissue and inflammatory response as compared with [^{11}C]Methionine.

[^{18}F]FMT as a metabolic indicator is useful for detecting tumors and monitoring response to therapy. Biochemically, FMT is a tyrosine analog that is not incorporated into nascent proteins; however, it does reflect amino acid uptake that is increased in cancer cells

(Ishiwata et al., 2004). In patients with non-small cell lung carcinoma, FMT uptake in primary adenocarcinoma was suggested to be an indicator of poor prognosis (Kaira et al., 2009). FMT has also been useful in identifying bone lesions (Ishiwata, et al., 2004). The D isomer of FMT was used to monitor squamous cell carcinoma in a mouse model system. Irradiated mice showed a decrease in uptake D-FMT after radiation in contrast to FDG and ^{11}C Met (Murayama et al., 2009). These results suggest that D-FMT may be a good indicator of early tumor response to treatment. The nucleotide analogs [^{11}C] thymidine and [^{18}F]FLT have also been used to detect malignancies. FLT is a thymidine analog that can be phosphorylated by thymidine kinase 1 which is elevated in proliferating cells (Rasey, et al., 2002). Phosphorylation traps FLT, resulting in its accumulation in cells. While FLT undergoes phosphorylation it is not clear if it is incorporated in DNA. Like FMT, FLT has been most useful in monitoring tumor response to therapy (reviewed in Barwick et al., 2009). In fact, FLT was similar to FMT in its ability to monitor early tumor response to radiation (Murayama, et al., 2009).

Both [^{11}C]Choline and [^{18}F]Choline, are indicators of phospholipid metabolism in cells. Choline is transported into cells where it is metabolized to phosphocholine by choline kinase, an enzyme that is frequently upregulated in tumor cells (Ramirez de Molina, et al. 2002 a,b). Phosphocholine is negatively charged and remains trapped within the cells. Labeled choline has been used extensively in prostate cancer studies. For example, an increase of ^{11}C choline in prostatic malignancies has been recently been shown to be an indicator of aggressiveness in prostate cancer patients (Piert et al., 2009). Several recent reviews discuss imaging in prostate cancer and the reader is directed to these reviews for further reading (Edmonds et al., 2009; Jadvar, 2009; Zaheer et al., 2009). Labeled choline, including ^{18}F-fluoroethylcholine in animal models, has also been used to detect hepatocellular carcinoma and brain tumors (Talbot et al., 2006; Kubota et al., 2006; Kolthammer et al., 2011).

Acetate is taken up by cells, converted to acetyl CoA and ultimately incorporated into the cell membrane (Howard & Howard, 1975). [^{11}C]Acetate has recently been used to detect increased glial tumor metabolism (Liu et al., 2006; Tsuchida et al., 2008). 2-^{18}F-fluoroacetate has been successful in detecting glioblastoma in a mouse model system (Marik et al., 2009). Like labeled choline, labeled acetate is also a useful tracer for prostate cancer. A review has recently been published by Jadvar (2011) comparing the use of labeled acetate versus FDG or labeled choline and discussing the utility of these imaging probes in prostate cancer detection.

2.2 Imaging of molecular complexes in living cells

Our knowledge about the function of molecular complexes and their subcellular localization has rapidly expanded with the development of a wide array of encoded fluorescent probes that have enabled direct observation of metabolic pathways in living cells (reviewed in Zhang et al., 2002). Several of these probes utilize fluorescence resonance energy transfer (FRET) to detect interaction between molecules or to assess cellular levels of metabolites and monitor their compartmentalization. Among such FRET probes that sense metabolites, "cameleon" measures intracellular calcium (Miyawaki et al., 1997), and other probes have been designed to non-destructively sense phosphorylation by specific kinases such as protein tyrosine kinases (Ting et al., 2001) or protein kinase A (Zhang et al., 2001).

Fluorescent nanosensors have also been developed for concentration-dependent sensing of maltose in yeast (Fehr et al., 2002), as well as for detecting glucose uptake and subcellular compartmentalization (Fehr et al., 2003; Fehr et al., 2005) or ribose uptake (Lager et al., 2003) in mammalian cells. Furthermore, bimolecular fluorescence complementation (BiFC) can detect interaction between protein partners in living cells. Individual proteins are fused with non-fluorescent fragments of green fluorescent protein (GFP; or one of its variants). If the two fusion proteins interact, this brings the GFP fragments in close enough proximity to reconstitute fluorescence, and imaging reveals subcellular localization of the complex (Hu et al., 2002). Multicolor BiFC allows detection of multiple complexes simultaneously in living cells, and it can be used to measure the efficiency of complex formation between a protein of interest and each of its known partners (Hu & Kerppola, 2003). One example for how BiFC can be used to monitor cell physiological readout for cancer pathways in single cells was recently demonstrated by visualizing activation of caspase-2, the initiator caspase for mitochondrial apoptosis pathway (Bouchier-Hayes et al., 2009). This is interesting in light of the evidence that caspase-2 is a tumor suppressor. Caspase -/- mouse embryonic fibroblasts (MEFs) resisted apoptosis, and they showed increased proliferation as well as tumor formation most likely compounded by lost function of p53 (Ho et al., 2009).

3. Nuclear organization and gene expression

3.1 Nuclear organelles

Organization of nuclear compartments can reflect metabolic status in mammalian cells. This is exemplified by numerous accounts of altered nuclear structure, nuclear organelles and nuclear biochemistry observed in a wide range of diseases. Tying altered gene expression to changes in nuclear organization is an area of intense current research (reviewed in Rajapaske and Groudine, 2011). Among the best examples of such observable alteration is seen in the perinucleolar compartment (PNC), a nuclear organelle found adjacent to the nucleolus that contains RNA binding proteins and is enriched with transcripts synthesized by RNA polymerase III (Huang et al., 1998). Metabolism of RNA polymerase III transcripts is suggested as a primary factor regulating PNC size (Wang et al., 2003). Variation in PNC size is medically relevant because the size of the PNC has been directly correlated with disease staging. Analysis of clinically staged breast cancer tissue samples showed that presence of PNCs increases with disease progression, such that metastatic tumors have the largest and most abundant PNCs (Kamath et al., 2005). Because PNC prevalence correlated with metastatic potential and malignancy in other solid tumors (Norton et al., 2008), PNC status can therefore be used as a simple and relatively low-cost prognostic marker for tumor progression. Further study is needed to determine if the PNC changes occur in other cancers, or if the changes are a result or a cause of cellular transformation. Regardless, defining the primary functions of the PNC and understanding the biochemical pathways housed in this nuclear organelle could lead to developing very specific tools for knocking out breast tumors and other types of tumors. Along these lines, an automated high-throughput imaging screen performed in living cells expressing fluorescently tagged PNC component polypyrimidine tract binding protein (PTB-GFP) identified compounds that disassemble the PNC (Norton et al., 2009). This work shows promise not only for cancer drug development, but also for the general assessment and screening of compounds that effect nuclear structural changes, as well as for scientists to determine where compounds

interfere with cellular biochemistry in order to better understand the metabolic pathways that regulate metastasis.

Despite decades of intense research, new players involved in protein coding gene expression are still being identified and characterized. Many of the factors localize to specific nuclear organelles such as Cajal bodies and nuclear speckles (also called SC35 domains or interchromatin granule clusters; reviewed in Spector and Lamond, 2010) Nuclear speckles are storage sites for pre-mRNA processing factors from which factors are exchanged with the nucleoplasm and recruited to nascent transcripts for co-transcriptional pre-mRNA processing (reviewed in Spector & Lamond, 2010). The organization of nuclear speckles reflects as well as impacts the global status of pre-mRNA synthesis and the efficiency of pre-mRNA processing. Inhibition of RNA polymerase II by alpha-amanitin supports the recruitment model, as this treatment causes RNA processing factors to remain in enlarged rounded nuclear speckles speckle rounding (Lamond & Spector, 2003). Disassembly of nuclear speckles distributes components throughout the nucleoplasm and alters pre-mRNA processing (Sacco-Bubulya & Spector, 2002). Purification of nuclear organelles has identified many of the nuclear proteins whose components are used for synthesis and processing of RNA (Mintz et al., 1999; Saitoh et al., 2004; Andersen et al., 2004) and is beginning to reveal functions for RNAs (Prasanth et al., 2005; Tripathi et al., 2010). Polyadenylated RNA was previously shown to be enriched in nuclear speckles by fluorescence in situ hybridization methods (Visa et al., 1993; Huang et al., 1994). As these new players are identified, synthetic gene reporter systems will be incredibly useful tools for determining the kinetics of their assembly at transcription sites, as well as to pin down their specific functions in gene expression.

Visualizing biosynthetic pathways in the nucleus has relied on a variety of experimental approaches. Labeling cellular structures or contents (e.g. lipids, mitochondria, DNA) non-immunologically with fluorescent molecules, or by using immunocytochemical approaches, has been described extensively elsewhere (Spector & Goldman, 2006). Incorporation of nucleotide analogs into nascent strands is a common way to label nucleic acids during their synthesis, and can be used to visualize entire chromosomes, individual DNA replication foci, or transcription factories. Nucleotide analogs can be radioactive, enzymatic, fluorescent or in some other way tagged for detection. Radioactive nucleotide incorporation relies on detection by autoradiography which has the disadvantage of requiring long exposure times of several months, offers low resolution, and is not typically the preferred labeling method. Recent technical advances using nucleotide incorporation approaches or various molecular tagging methods, as well as advances in super-resolution imaging systems (Huang et al., 2009), are certain to continue rapidly expanding our knowledge about localization and kinetics of nuclear pathways.

3.2 Transcription and RNA processing

BromoUTP incorporation is a widespread tool for to labeling transcription sites in situ. Cells are gently permeabilized to allow uptake of nucleotide analog, followed by incubation in a transcription buffer cocktail that promotes elongation of nascent transcripts (Haukenes et al., 1997). A short pulse of labeling in mammalian cells (~5-8 minutes at 37 degrees Celsius for HeLa cells) is sufficient to globally label nascent RNA, which can be subsequently detected in transcription foci throughout the nucleus corresponding to RNAs synthesized

predominantly by RNA polymerases II and III (Sharma et al., 2010; Sacco-Bubulya and Spector, 2002) or specifically in nucleoli corresponding to ribosomal RNAs synthesized by RNA polymerase I (Dundr et al., 2002). Fluorouridine is also useful for labeling transcription sites in situ (readers should note that this is halogenated, not fluorescent), and has the advantage that it directly enters the cells without the need for detergent permeabilization (Boisvert et al., 2000). In addition, permeabilizing cells using different detergents and various permeabilization times can affect labeling efficiency, for example, in nucleolar transcription foci versus transcription foci in the nucleoplasm, and nucleotide analogs appear to be incorporated with different degrees of efficiency in distinct nuclear compartments in mammalian cells (P. Bubulya, unpublished observations). RNAs synthesized by distinct RNA polymerases I, II or III have been shown in dedicated nuclear transcription "factories" by sophisticated twists on BrUTP incorporation methods (Pombo et al., 1999). Regardless, in all these studies the cells are not alive; following pulse labeling, removal of excess nucleotide, and fixation of the cells, nucleotide incorporation is detected by subsequent labeling of the halogenated nucleotide. Studies in living cells have revealed information regarding the kinetics of transcription and processing machinery assembly and transcript elongation rates for RNA polymerases I and II (Dundr et al., 2002; Kimura et al., 2002) as well as the cell cycle regulated assembly of the nuclear organelles that house these machineries.

The synthesis and processing of ribosomal RNAs for ribosome production is the most robust gene expression pathway in mammalian cells. Although products of all RNA polymerases are required for final assembly of ribosomes, ribosome biogenesis occurs in nucleoli (reviewed in Leary & Huang, 2001, Hernandez-Verdun et al., 2002). Activation of the rRNA genes evades global inhibition of transcription on condensed chromosomes during mitosis. Imaging methods have demonstrated the involvement of pre-rRNAs in the onset of nucleolar organization at daughter cell chromatin and that different chromatin regions containing rRNA genes come together during nucleolar maturation (Hernandez-Verdun et al., 2002). Ribosomal components are delivered to newly forming nucleolar bodies as they are needed for pre-rRNA processing, and individual components show rapid exchange in and out of these bodies during assembly (Dundr et al., 2000). During interphase, individual components of RNA polymerase I enter nucleoli independent of others, and different incorporation efficiency and nucleolar residence time for independent components suggests sequential assembly of complexes with each round of transcription (Dundr et al., 2002).

Reporter gene constructs that have been engineered and stably integrated into cellular genomes to visualize RNA polymerase II transcription in living cells made it possible to study the position and activity of transcription sites in real time, in the context of chromatin structure and global nuclear organization. Tandem arrays of DNA containing repeated binding sites for fluorescently-tagged DNA binding proteins can be stably integrated into chromatin where they become assembled into higher order chromatin structure. These arrays have been used to visualize chromatin dynamics, and they have been coupled to reporter genes downstream to visualize the kinetics of chromatin unfolding and transcript synthesis in living cells (reviewed in McNally, 2009 and in Rafalska-Metcalf & Janicki, 2007).

In one system, integrated tandem arrays of mouse mammary tumor virus driving a *ras* reporter (called MMTV-LTR-ras-BPV) created a specific locus containing 800-1200 binding

sites for gluococorticoid receptor (GR). Following controlled expression of a GFP-tagged-GR (GFP-GR) and hormone addition, GR nuclear translocation as well as GR-GFP recruitment to the locus were observed in living cells (McNally et al., 2000). Photobleaching techniques determined that GFP-GR undergoes its rapid exchange on locus chromatin (McNally et al., 2000). Reporter transcripts were detected by RNA fluorescence in situ hybridization (RNA-FISH) in the vicinity of GR-GFP accumulation on the reporter locus. Since GR-GFP reflected the underlying DNA binding sites on chromatin, the extent of locus chromatin decondensation could in turn be observed and correlated with the amount of transcription at that site (Muller et al., 2001).

Other systems have been developed to allow visualization of chromatin independent of transcription activity, such that both the transcriptional inactive and transcriptionally active chromatin can be observed, and the transitions between these two states can be studied. One such system consists of a tandem array of synthetic reporter sequences in baby hamster kidney (BHK) cells containing lac operator repeats proximal to an inducible reporter gene encoding cyan fluorescent protein that is targeted to peroxisomes via a serine-lysine-leucine tag (SKL; Tsukamoto et al., 2000). Expression of fluorescently-tagged lac repressor protein in these cells labels the locus chromatin, and inducing transcription allows live observation of the transition from the inactive to active state, revealing continued chromatin decondensation at the locus over the time course of imaging (Tsukamoto et al., 2000). While reporter transcripts could be detected in that system by RNA-FISH, this method does not allow real time imaging of transcripts. The same basic reporter system was then further developed such that transcripts would contain MS2 bacteriophage viral replicase translational operator sequences (MS2 stem loops). These sequences are recognized by the MS2 coat protein that can be fluorescently tagged and expressed in the reporter cells for imaging transcription in real time (Janicki et al., 2004). This latter system, referred to as U2OS 2-6-3, enables visualization of DNA, RNA and protein product for a single reporter locus in living cells (Janicki et al., 2004). For that reason, it is a very powerful tool for understanding all the steps of RNA polymerase II-mediated gene expression in any phase of the cell cycle. For example, the transition from condensed to decondensed chromatin, the switch from heterochromatic to euchromatic modifications, the exchange of histones H3/H3.3, the recruitment of transcription factors, the kinetics of reporter transcript production, and the recruitment of pre-mRNA processing machinery were all observed in living cells (Janicki et al., 2004). A further advantage of these live cell reporter systems for transcription is that the lac repressor can be fused both with a fluorescent protein AND a second protein/domain that sequesters it to a particular subnuclear compartment. For example, fusing lac repressor to the fluorescent protein mCherry and to lamin B1, and placing the expression of this triple fusion protein under the control of an inducible promoter, allowed for inducible tethering of a reporter locus to the nuclear periphery (Kumaran and Spector, 2008). Despite that the peripheral regions of the nucleus are generally thought of as heterochromatic and transcriptionally silent, this reporter locus retained its transcriptional activity upon relocation to the nuclear lamina. This tethering system opens many possibilities for gaining both spatiotemporal and kinetic information about gene expression events occurring on a gene locus during its nuclear repositioning, as well as a novel way to test how nuclear compartments in diseased cells might demonstrate misregulated gene expression pathways (Kumaran and Spector, 2008).

Many continuing studies are using such systems to learn more about transcription regulation and how it relates to nuclear dynamics or RNA trafficking. The involvement of proteins and RNAs in pre-mRNA synthesis and processing, or on chromatin condensation/decondensation during gene regulation can be systematically evaluated either by RNAi-mediated knockdown or overexpression studies. For example, depletion of 7SK RNA resulted in upregulation of transcription on a modified version of the U2OS 2-6-3 gene reporter system (Prasanth et al., 2010). Furthermore, the transcripts themselves can be observed directly in living cells. Individual mRNP particles can be tracked to gain information about mRNP movement through the nucleoplasm by using the U2OS 2-6-3 cells (Shav-Tal et al., 2004). Photoactivation studies showed the single mRNPs freely diffused in all directions as they moved away from the transcription site. In addition, general inhibitors of cellular metabolism surprisingly caused a decreased mobility of mRNP particles, mostly likely explained by overall restructuring of nuclear organization under these conditions, that was restored when energy levels were reset to normal (Shav-Tal et al., 2004). At least one new system has been recently developed for examining the trafficking of mRNA (and visualizing its protein product) that is certain to shed light on the spatial and temporal kinetics of synthesis, export, and cytoplasmic transport of mRNA to its cellular location where it is ultimately translated into protein (Ben-Ari, et al., 2010). Our capability for RNA imaging is on the verge of complete transformation due to a newly developed tool for tagging and imaging RNAs in living cells (Paige et al., 2011). This new "RNA version" of green fluorescent protein, called Spinach, was successfully used to image the dynamics of 5S ribosomal RNA in living cells. Spinach as well as other RNA-tagging molecules in a range of excitation and emission spectra will undoubtedly open new avenues for discovery with potential applications in RNA-RNA and RNA-protein FRET (Paige et al., 2011).

Pre-mRNA splicing regulation can be studied by using minigene reporters that typically contain only a small segment of genomic DNA from a given gene subcloned into a mammalian expression vector. A well-characterized beta-tropomyosin (BTM) minigene (Helfman et al., 1988; Huang & Spector, 1996) was stably integrated into HeLa cells (Sacco-Bubulya and Spector, 2002; Sharma et al., 2011). The BTM minigene is useful for monitoring constitutive as well as alternative splicing of minigene transcripts. One advantage of expressing transcripts from reporter minigenes is that a comparison can be made between precursors versus spliced transcripts produced at the reporter locus that can be visualized by RNA-FISH (Sacco-Bubulya and Spector, 2002; Sharma et al., 2011). Altered splicing of the reporter transcript can be detected after various treatments, for example, RNAi-mediated depletion or overexpression of specific splicing factors. The extent of reporter transcript processing can be monitored in situ. Sharma et al. (2011) recently demonstrated that HeLa cells treated with siRNA duplexes targeting the splicing factor Son showed increased skipping of exon 6 in BTM transcripts both by quantitative PCR and by RNA-FISH. In addition, a genome-wide screen identified human transcription and splicing targets for Son that include chromatin modifiers and cell cycle regulators (Sharma et al., 2011).

Many of the above mentioned reporter gene loci have been instrumental in studying the onset of pre-mRNA synthesis and processing in post-mitotic nuclei. Somewhat similar to what was observed during nucleolar reassembly following mitosis, sequential nuclear entry of pre-mRNA synthesis and processing factors also occurs in an ordered sequence according to the timing for when factors are needed (Prasanth et al., 2003). Interestingly, transcription

factors and RNA polymerase II are detected in daughter nuclei first, followed by RNA processing factors. Only after all components are available in the nucleus for coupled RNA synthesis and processing are the elongating RNA polymerase II and exon junctions in reporter mRNAs detected (Prasanth et al., 2003). Recently, live cell imaging demonstrated that specific factors regulate post-mitotic reinitiation of transcription (Zhao et al., 2011). Active transcription sites are remembered, or "bookmarked", by histone post-translational modification, specifically by histone H4 lysine 5 acetylation. This mark persists on transcriptionally inactive mitotic chromatin. Bromodomain protein 4 (BRD4) recognizes this mark to increase kinetics of RNA polymerase II transcription on a reporter locus following mitosis as compared to interphase cells (Zhao et al., 2001).

4. DNA replication

5-Bromodeoxyuridine (BrdU) incorporation is a common way for observing DNA replication in situ, and it is useful for general labeling of nuclear replication foci. Typically a pulse of BrdU is administered to live synchronized cells, and localization of the BrdU is observed after preserving cells by fixation and immunofluorescence labeling with antibodies against BrdU to show progression of replication in different subnuclear regions over time (Nakamura et al., 1986). This approach proved to be substantially better than radioactive nucleotide incorporation for replication labeling due to limited spatial resolution of autoradiography. Perhaps continued development of more sensitive and faster methods to incorporate nucleotide analogs will open new avenues for DNA replication studies. One such approach employs 5-ethenyl-2′-deoxyuridine incorporation followed by detection using "click" chemistry. This approach is useful in that it can be done in live cells or tissues to label cells in S-phase and also for direct imaging and high-throughput applications, although the cytotoxicity of the reaction prevents long-term cell survival (Salic & Mitchison, 2008). All these types of approaches have been used widely to label replicating DNA.

The overall DNA replication pattern changes progressively through S-phase as different nuclear regions undergo DNA replication at different times, and this generally correlates with chromatin status such that euchromatin replicates earlier in S-phase than heterochromatin (O'Keefe et al., 1992). Labeling of replication foci has been done in a variety of cell types and with multiple different labeling methods that commonly rely on labeled nucleotide analog such as bromo- or biotinylated-dUTP, and allow for comparison of replication foci to other cellular structures (Nakayasu & Berezney, 1989). Similarly in live cell analysis by GFP-PCNA labeling, one can see that replication factories in live cells show limited subnuclear movement, and each individual replication site arises and recedes independently from others (Leonhardt, 2000). The global position of chromosome organization in interphase nuclei and through mitosis has been analyzed after tagged-dUTP incorporation as well as "chromosome painting" methods. Such approaches have shown that the gene density of chromosomes may position them differently in nuclear space. Gene-dense chromosomes more frequently appear in the nuclear interior, while gene-poor chromosomes are found at the nuclear periphery (Bolzer et al., 2005; Spector 2003). In addition, the timing of replication for specific gene loci has been determined by coupling fluorescence in-situ hybridization methods with the nucleotide analog incorporations. For example, alpha-satellite DNA at centromeres replicates at mid-S-phase in a variety of human cell types (O'Keefe et al., 1992). Also, active rDNA replication foci labeled by EdU

pulse became activated early in S-phase, while inactive rDNA repeats replicated later (Dimitrova 2011). Early replicated rDNA repeats were then repositioned to the nucleolar interior where they coincided with RNA polymerase I transcription factor UBF, indicating that these replicated regions are transcriptionally active rDNA genes (Dimitrova 2011).

After completion of a number of cell cycles following tagged-dUTP incorporation, the number of labeled chromosome territories eventually decreases by successive distribution to daughter nuclei such that individual chromatin territories are labeled and can be compared relative to other objects or gene loci. Fluorescently tagged nucleotide (e.g. Cy5-dUTP) is also useful for imaging DNA strand condensation into chromosomes and to monitor chromosome organization through mitosis (Manders et al., 1999). Chromosome painting techniques in fixed cells have supported the model that individual chromosomes occupy discrete nuclear regions during interphase (Cremer et al., 1993). A significant advance in understanding chromatin dynamics was made with histone H2B-green fluorescent protein (GFP), which incorporates into mononucleosomes without disturbing the cell cycle (Kanda et al., 1998). H2B-GFP labeled chromatin was originally used to follow the position and segregation of double minute chromosomes during mitosis (Kanda et al., 1998). Widespread use of H2B-GFP in many subsequent studies has given more global insight into the organization and dynamics of chromatin. H2B-GFP observation in living HeLa cells has shown that chromosomes maintain nuclear position or "neighborhoods" through interphase, but become repositioned to new neighborhoods following mitosis (Walter, 2003). In other studies, mitotic chromosomes were demonstrated to have remarkably ordered positioning suggesting that chromosome position is transmissible (Gerlich et al., 2003). Photobleaching experiments showed clearly that a region of YFP-H2B chromatin located at the extreme end of an interphase nucleus would become distributed to many subnuclear regions distributed throughout post-mitotic daughter nuclei (Walter et al., 2003). Because spindle tension draws sister chromatids of adjacently situated chromosomes at the metaphase plate to roughly the same regions of the daughter cell, there is relative symmetry of chromosome arrangement in daughter nuclei. However, the pattern of chromosome territories in daughters is not similar to the pattern of the mother cell, and overall chromosome territory pattern appears to rearrange significantly with each cell cycle (Walter et al., 2003).

Temporal control of replication timing must be reinitiated with each cell cycle, and many questions regarding how the timing and organization is controlled remain unanswered (reviewed in Lucas & Feng, 2003). It will be particularly interesting to follow the activity of specific replication proteins through the cell cycle, or to know what specific nuclear structures/complexes might support dynamic assembly of replication foci. Basic research in this regard will be crucial for us to understand the mechanisms that underlie observed differences in replication of specific gene loci in diseased cells versus normal cells. For example, malignant cells demonstrated less synchrony in replication of homologous loci, having early and late replicating alleles rather than synchronously replicating alleles found in normal cells (Amiel et al., 1998). Also, the spatial organization of DNA replication foci within the nucleus during early S-phase, and the association of these replication foci with intranuclear lamin A/C was altered in immortalized cells (Kennedy et al., 2000). An understanding of how replication of a gene locus is tied to its transcriptional activity may also shed light on why replication organization and timing is altered in disease. In order to

address such questions, it will be important to analyze cell cycle dependent events in large numbers of cells. A very promising new technique for measuring cell cycle dependent growth was demonstrated recently, using spatial light interference microscopy (SLIM) coupled with a fluorescence marker for S-phase to analyze cell cycle phase within a cell population (Mir et al., 2011). The applications for this technique to a range of cell types, as well as to microscopy systems that utilize multi-channel fluorescence imaging, open endless possibilities for developing variations on this method to image cellular metabolism in the context of cell growth even within a complex cellular population.

5. Conclusion

The rapid progress recently made toward developing metabolic tracer molecules shows great promise for new applications in clinical diagnostics. Further characterization of novel imaging probes is needed to understand how they can be used to image and identify malignant tissues. Rapidly screening novel tracer molecules for efficacy in identifying tumors in cell culture systems, animal models and clinical trials is a crucial ongoing challenge aimed toward building a battery of tools for imaging cancer metabolism in patients. Feeding into clinical studies is a vast amount of knowledge gained from basic research characterizing metabolic pathways in single cells. This information has potential for wide use for diagnostic imaging, but awaits further research and development into translational medicine that will utilize novel biomarkers and imaging technologies. Finally, continued development of super-resolution imaging platforms for both basic research and clinical use are certain to have a major impact on our understanding of molecular complexes, especially with regard to colocalization of specific protein-protein, protein-RNA or protein-DNA complexes within the overall context of cellular architecture.

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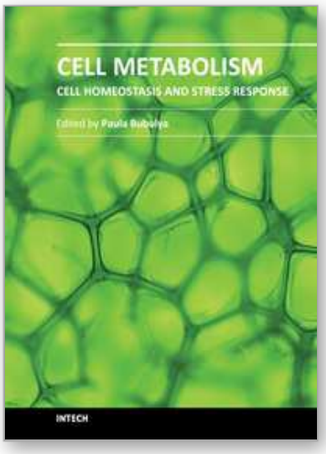

Cell Metabolism - Cell Homeostasis and Stress Response

Edited by Dr. Paula Bubulya

ISBN 978-953-307-978-3

Hard cover, 208 pages

Publisher InTech

Published online 25, January, 2012

Published in print edition January, 2012

A global research community of scientists is teasing out the biochemical mechanisms that regulate normal cellular physiology in a variety of organisms. Much of current research aims to understand the network of molecular reactions that regulate cellular homeostasis, and to learn what allows cells to sense stress and activate appropriate biochemical responses. Advanced molecular tools and state-of-the-art imaging techniques discussed in this book continue to provide novel insights into how environmental changes impact organisms, as well as to develop therapeutic interventions for correcting aberrant pathways in human disease.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Athanasios Bubulya and Paula A. Bubulya (2012). Imaging Cellular Metabolism, Cell Metabolism - Cell Homeostasis and Stress Response, Dr. Paula Bubulya (Ed.), ISBN: 978-953-307-978-3, InTech, Available from: http://www.intechopen.com/books/cell-metabolism-cell-homeostasis-and-stress-response/imaging-cellular-metabolism

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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
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