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Fluorescence Sheds Light on DNA Damage, DNA Repair, and Mutations

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Abstract

DNA damage can lead to carcinogenic mutations and to toxicity that promotes diseases. Therefore, having rapid assays to quantify DNA damage, DNA repair, mutations, and cytotoxicity is broadly relevant to health. For example, DNA damage assays can be used to screen chemicals for genotoxicity, and knowledge about DNA repair capacity has applications in precision prevention and in personalized medicine. Furthermore, knowledge of mutation frequency has predictive power for downstream cancer, and assays for cytotoxicity can predict deleterious health effects. Interestingly, tests for all of these purposes have been rendered faster and more effective via adoption of fluorescent readouts. Here we provide an overview of established and emerging cell-based assays that exploit fluorescence for studies of DNA damage and its consequences.

Keywords

DNA damage; DNA repair; Comet Assay; gamma H2AX; Fluorescence assays

Implications of DNA Damage and Repair.

DNA damaging agents are ubiquitous. They are present in the environment (e.g., sunlight and air pollution), they can be found in the food and water (both as naturally occurring chemicals and as contaminants), and they are often used at high levels to treat cancer. Fortunately, numerous DNA repair pathways exist to protect cells from DNA damage [1, 2]. Nevertheless, DNA repair leaves some DNA damage unrepaired, resulting in hundreds of DNA lesions that can interfere with transcription and with accurate copying of the genome, promoting cancer and other diseases (see Box1)[3–5]. Importantly, the ability to repair DNA varies across populations [6, 7], and differences in DNA repair capacity are increasingly appreciated as an important factor in modulating the risk of cancer [8–10]. Given the importance of DNA damage, we need effective DNA damage sensors, DNA repair ‘meters,’

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mutation detection strategies, and tests for DNA damage-induced cytotoxicity. Such technologies could be used for identifying chemicals that have the potential to damage DNA, for identifying people with reduced DNA repair capacity, for optimizing chemotherapy and for predicting the health consequences of exposures. Here we describe key fluorescence-based live-cell assays that can be used for each of these applications (Figure 1).

Importance of cell-based phenotypic assays for detecting DNA damage and repair.

One indirect approach for assessing a person's DNA repair capacity is to identify single nucleotide polymorphisms (**SNPs**) (see Glossary) in DNA repair genes. However, most phenotypic consequences of SNPs remain unknown [11], and the impact of a SNP can be context-dependent. As an example, it is critical for activities in the base excision repair (BER) pathway to be balanced [12, 13]. If the first step in the pathway is accelerated by a SNP, this may be beneficial for one person, for whom the downstream steps are rapid, but problematic for another person, for whom there can be a buildup of toxic BER intermediates, such as strand breaks. A cell-based assay integrates the contributions of all SNPs that contribute to overall pathway efficiency.

Another way to assess DNA repair capacity is to use systems level approaches, such as transcriptional profiling, proteomics, and genome wide genotyping [14–17]. Although these unbiased methodologies are powerful and increasingly cost-effective, it is noteworthy that they are a proxy for cell behavior, and cannot replace direct measures of cellular phenotypes. Indeed, the pathways that are involved in the response to, and repair of, DNA damage are subject to regulatory mechanisms at the level of transcription, translation, post-translational modifications, protein localization, and chromatin structure, limiting efficacy of analysis of any single biological process for predicting function.

Cell-based assays that leverage fluorescence for visualization of DNA damage, cytotoxicity and mutations

To address the need for cell-based assays for DNA damage and it's consequences, several fluorescence-based analytical methods have been developed, and these fall into five categories: a) analysis of subnuclear DNA repair foci; b) analysis of repair of site-specific DNA lesions in plasmids; c) analysis of DNA strand breaks; d) analysis of cytotoxicity; and e) analysis of mutations.

Analysis of subnuclear DNA repair foci

Chromatin adjacent to double-stranded breaks (DSBs) is modified in a manner that can be detected and exploited for measuring their repair [18, 19]. For example, following formation of a DSB, a rapid wave of phosphorylation at serine 139 converts histone variant H2AX to γ-H2AX in the vicinity of the break over a region that is large enough to be detected by immunocytochemical methods [20]. As such, DSBs can be directly visualized and quantified by measuring the number of fluorescent subnuclear foci that are revealed by

immunofluorescence. In addition, DNA repair proteins are recruited to the sites of damage [19, 21, 22]. As concentrations of repair proteins increase at or near sites of DNA damage, repair foci can be visualized using immunofluorescence. Indeed, there are promising studies utilizing super-resolution microscopy to visualize repair foci [23].

There have now been hundreds of studies of the dynamics of chromatin modification and repair proteins, and these studies both contribute fundamentally to our understanding of DNA repair, while also providing a useful tool for applied research questions. Although the resolution of repair foci is not perfectly in sync with physical rejoining of DSBs [24], it is nevertheless possible to rapidly screen people for their capacity to repair DSBs by performing a time course for the disappearance of γ-H2AX foci [25]. As such, Rapid Automated Biodosimetry Technology (RABiT) has been developed to measure γ-H2AX fluorescence in a high-throughput fashion using a fingerstick sample of blood [26–28]. In its first incarnation, the RABiT approach incorporated in-house technologies for robotics and it included a radiation source to deliver damage and enable quantification of the damage in an automated fashion from damage induction to fluorescence image acquisition. Presently, researchers are exploiting cutting edge high-throughput platforms for measuring individual global DNA repair capacity [26] and there have also been advances that leverage high-throughput imaging linked to flow cytometry [29]. DNA repair capacity modulates the risk of cancer development and other diseases (see Box 1). Since thousands of blood samples can be analyzed in a matter of hours, these high-throughput methods for γ-H2AX foci quantification are particularly valuable in molecular epidemiology aimed at uncovering new connections between DNA repair and diseases.

Analysis of Repair of site-specific DNA lesions in plasmids

The development of the fluorescence-based multiplexed host cell reactivation assay (FM-HCR) assay represents a significant advance in cell-based assays for directly measuring DNA repair efficiency. Host cell reactivation (HCR) technology was first developed and named for the analysis of host cells reactivating viruses that had been treated with radiation [30]. More recently, HCR assays have been adapted to measure the repair of plasmids that are exposed to agents that induce transcription blocking DNA damage [6, 31]. DNA repair removes the transcription blocking damage, and can therefore be detected by measuring reactivation of expression of a reporter gene. Early reporter genes encoded enzymes such as chloramphenicol acetyltransferase [32], and more recently luciferase and finally fluorescent proteins [33, 34]. While many exciting discoveries have been made using the traditional HCR assay, two critical weaknesses were that it was non-specific (most DNA damaging agents create a spectrum of DNA lesions), and that it was cumbersome (in some cases requiring complicated and slow biochemical assays to detect recovery of transcription). Fluorescence-based HCR assays with site-specific DNA lesions have thus been developed for DNA repair pathways wherein repair can be detected by a change in the sequence of the transcribed DNA [35, 36]. However, strategies for simultaneously measuring the activities of multiple repair pathways, and for measuring repair of DNA lesions that do not block transcription, and whose repair does not entail a change in the DNA sequence were not available. Together, Samson and Nagel overcame these limitations. First, they expanded HCR assays to include DNA lesions that are bypassed by RNA polymerase by exploiting

transcriptional mutagenesis, the error-prone bypass of unrepaired DNA lesions by RNA polymerase [37]. This opened the door to monitoring repair of site-specific DNA lesions that do not block transcription, providing a leap ahead in the value of the assay. Second, to achieve multiplexing and to normalize for transfection efficiency, they exploited expression cassettes for multiple fluorescent proteins. As one example, expression of EGFP is inhibited by an abasic site analog, tetrahydrofuran, and when that site is repaired, expression of EGFP rises [6]. By normalizing EGFP expression to expression of a co-transfected plasmid expressing another color (in this case tagBFP), it is possible to control for transfection efficiency. To monitor a cell's ability to repair the abasic site, normalized expression of the lesion-containing EGFP reporter is compared to normalized expression of an undamaged EGFP reporter measured in a separate transfection. This approach has been extended to include over a dozen different substrates that reflect numerous DNA repair pathways, offering unrivaled specificity and throughput for analyzing DNA repair capacity [31]. FM-HCR has recently been used to measure DNA repair capacity for multiple repair pathways in human cells and has been shown to predict glioblastoma responsiveness to treatment with DNA damaging chemotherapy drug, temozolomide [38]. Given that many chemotherapeutics are DNA damaging agents, this work points to the promise of DNA repair functional assays as predictors of the effectiveness of cancer therapy more broadly, with applications in personalized medicine and patient stratification.

Analysis of DNA Strand Breaks

The comet assay (or single cell gel electrophoresis assay) affords a rapid and straightforward approach for quantifying strand breaks in cell nuclei [39–41]. Briefly, cells are embedded in agarose, lysed, and subjected to high pH to denature DNA. Subsequently, the resulting cell nucleoids are subjected to electrophoresis and for cells that harbor strand breaks, the DNA migrates more readily, leading to formation of what appears to be a comet tail. The alkaline method quantifies single-strand breaks (SSBs), abasic sites, and alkali sensitive sites that are converted into SSBs at high pH. A similar assay using neutral conditions quantifies DSBs. Although in theory the alkaline method could detect DSBs, for almost all exposures, the frequency of SSBs is at least an order of magnitude higher than the frequency of DSBs (for example, 1 Gy of gIR induces ~20-40 DSBs and ~1000 SSBs [42, 43]), and so it is generally appreciated that the alkaline method primarily detects SSBs.

The comet assay is actually quite versatile, and it can be modified for the purpose of detecting base damage. Under standard alkaline conditions of the comet assay, damaged bases do not affect DNA migration and therefore cannot be detected. However, addition of enzymes that recognize base damage and cleave the DNA is an effective approach for revealing the presence of base lesions [44, 45]. Commonly, this method has made use of the formamidopyrimidine-DNA glycosylase (FPG), which recognizes and nicks the DNA at sites of 8-oxoguanine and other purine oxidation products, thus converting undetectable base damage into detectable strand breaks. Additional enzymes used in this fashion include the human 8-oxoguanine DNA glycosylase OGG1, T4 Endonuclease V (which introduces strand breaks at cyclobutane pyrimidine dimers), and Endonuclease III (which introduces strand breaks at sites of oxidized pyrimidines)[46].

Although effective in principle, in reality the traditional comet assay suffers from being low throughput, laborious, and prone to bias. Two approaches have been developed to overcome these limitations: (i) The first is to enable more samples to be analyzed per unit area. For the traditional assay, a single microscope slide is required for each sample. Recent advances have given rise to methods for sample separation that enable analysis of multiple conditions on a single glass slide [47, 48]; (ii) The second approach is to use cell array technology referred to as the "CometChip" [49–52]. For the CometChip, microwells that are approximately the diameter of a single cell are arranged in an array, and cells are loaded by gravity. After processing using standard comet assay conditions, a comet microarray can be created. Advantages of this approach are that i) there is a reduction in overlapping comets, thus reducing bias caused by selection, ii) the number of comets per unit area is maximized, which enables more samples per unit area, iii) the method is compatible with the design of a standard microtiter plate (*e.g.*, 96 samples can be processed in parallel), and iv) analysis can be fully automated [50, 51, 53].

The HepaCometChip was developed to bring together the assets of the CometChip, namely metabolically competent cells such as hepatocytes with Cytochrome P450 activity, and DNA damage detection via repair trapping [54]. While generally beneficial, there are situations where p450-dependent metabolic processing converts an otherwise benign chemical into a DNA reactive chemical [55, 56]. As such, the traditional comet assay would miss detecting lesions that require metabolic activation unless target cells express p450 enzymes. To achieve this, HepaRG™ cells have been incorporated into the CometChip platform [57]. While this is a step closer to the desired sensitivity range, there remains an additional challenge: bulky lesions do not affect DNA migration and so cannot be detected using standard comet assay conditions. One way to overcome this limitation is to adapt the principle described above, which is to convert base damage into strand breaks. This can be accomplished by taking advantage of the fact that Nucleotide Excision Repair (NER) cleaves the DNA backbone upstream of bulky lesions prior to repair synthesis to close the gap [58]. Using inhibitors of NER that block repair synthesis, persistent single-strand breaks accumulate in proportion to NER activity [59, 60]. This approach overcomes a blind spot in screens for chemical safety and it is currently being validated to enable more broad utility. In addition to its potential use in identifying genotoxic chemicals, the comet assay has also been successfully utilized to asses DNA damage and to measure repair in tumor cells from patients with various types of cancer [61], and it has been exploited to measure the sensitivity of various types of tumors to radiation. These studies point to the promising application of the improved higher throughput versions of the comet assay in cancer studies and personalized medicine.

Analysis of cytotoxicity

In cancer research cell survival assays are important for predicting the efficacy of chemotherapy drugs to target and kill cancer cells, and in public health research cell survival assays have utility in screening for adverse effects of chemicals. Two of the most accurate methods to assess cell survival are the colony formation assay (which measures the ability of single cells to form colonies) and CellTiter-Glo (which uses the levels of ATP in the cell as an indicator that a cell is living). While the colony forming assay is effective, counting

colonies by eye is low throughput and vulnerable to bias. Cell-Titer Glo offers an unbiased high throughput approach, however ATP levels can be affected by processes that are independent of cell death. In addition, the assay is vulnerable to large differences in data output depending on the initial cell seeing density. Recently, the MicroColonyChip (μCC) was developed to overcome these limitations [62]. The μCC involves arraying cells in microwells and allowing the cells to grow and form microcolonies that are then quantified using fluorescence microscopy. The change in the distribution in microcolony size is used as the metric to directly query cell survival. The assay is resistant to variations caused by initial cell seeding density, and the microcolonies can be analyzed in an automated manner within only a few days (which prevents bias and greatly increases throughput compared to the gold-standard colony forming assay). This assay has the potential to be useful in cancer research to screen for drugs that will be effective in inducing toxicity to cancer cells, and it can be applied in high throughput screening for chemical safety testing.

Analysis of Mutations in vivo

Approaches have also been developed that exploit fluorescence as a readout for *in vivo* mutation assays. In particular, mouse models have been developed to detect DNA damage-induced sequence rearrangements *via* sensing homologous recombination (HR) events. To accomplish this, fluorescent yellow direct repeat (FYDR) mice harbor a direct repeat. The 5' expression cassette lacks an essential coding sequence on the 5' end of the cDNA, and the downstream expression cassette lacks the essential coding sequence at the 3' end of the cDNA. Recombination between the cassettes can restore full length EYFP, giving rise to fluorescent cells that can be detected *in situ* [63]. An analogous approach was used to create the Rosa26 Direct Repeat (RaDR) mice, and an advantage of this system is that the expression cassette was targeted to the highly expressed Rosa26 locus, enabling detection of fluorescent recombinant cells in most cell types [64]. More recently, another mouse model using EGFP expression was generated to measure homology-directed repair following induction of DSBs by an inducible endonuclease [65]. All of these mouse models have key advantages over previous mouse models and over sequencing. First, mutations can be analyzed by simple imaging, which is not challenging technically and is inexpensive. Second, mutant cells can be detected within their normal tissue context, yielding valuable information about cell identity and context [64, 66]. This information is lost using previous mouse models and sequencing for which tissue is destroyed to extract DNA. Finally, fluorescent labeling of mutant cells enables lineage tracing [67]. As such, conditions that enable clonal expansion can be detected. Since tumors evolve via successive waves of clonal expansion and accompanying mutagenesis, it is as important to understand physiological conditions that promote clonal expansion as it is to understand conditions that lead to mutations. This is an exciting new application of these mouse models that promises to bring a fresh perspective on underlying drivers of cancer.

Concluding remarks.

Fluorescence methods for detecting and quantifying DNA damage, repair, mutations, and cytotoxicity have myriad applications. Better assays for detecting DNA damage and cytotoxicity have relevance to the pharmaceutical industry, where there is a need to ensure

that drugs are safe and effective. Assays for DNA damage are also important for identifying industrial and environmental chemicals that have the potential to promote carcinogenic mutations. Having high throughput assays for DNA repair capacity has utility for many basic research applications, including enabling screens for novel genes that impact DNA repair [68, 69]. DNA repair assays are also promising as indicators of tumor responsiveness, with utility for individual patients as well as for identifying groups of patients most likely to benefit from a particular chemotherapeutic agent. Finally, having better ways to visualize mutant cells opens doors to knowledge of environmental and genetic factors that modulate susceptibility to DNA damage-induced mutations that drive cancer (see Outstanding Questions). Together, the fluorescence-based live-cell assays presented here open doors to safer drugs, a safer environment, personalized medicine, precision prevention, and new understanding of gene-environment interactions that modulate cancer susceptibility.

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Glossary

Single Nucleotide Polymorphism (SNP)
A common variation at a single nucleotide in DNA sequence (> 1% prevalence)

Base Excision Repair (BER)
Removes damaged DNA bases

Fluorescence-based Multiplexed Host Cell Reactivation (FM-HCR)
Using flow cytometry, measures the ability of cells to repair specific types of DNA damage using plasmid reporters with fluorescent readouts

Double Strand Breaks (DSB)
DNA lesions where both strands of the DNA helix are broken, leading to a double strand end

Rapid Automation Biodisometry Technology (RABiT)
Fully automated high throughput robotically based biodisometry designed to rapidly estimate individual dose of radiation following radiological event

Nucleotide Excision Repair (NER)
Removes mostly bases with large DNA adducts, such as those induced by UV light

Rosa26 Direct Repeat (RaDR)
Methodology to detect cells that have undergone an HR event in a transgene that gives rise to a fluorescent signal

Homologous Recombination (HR)

Repair processes wherein broken DNA is fixed via alignment of similar sequences, often leading to an exchange of sequence information between two helices

Abasic (AP) sites
Deoxyribose lacking a purine or a pyrimidine base. Also known as apurinic/apyrimidinic (AP) sites

HepaRG™
Terminally differentiated hepatocyte-like cells that are derived from hepatic progenitor cell line and that have many metabolic processes in common with the liver

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Box 1:

Role of DNA Damage and Repair in Disease

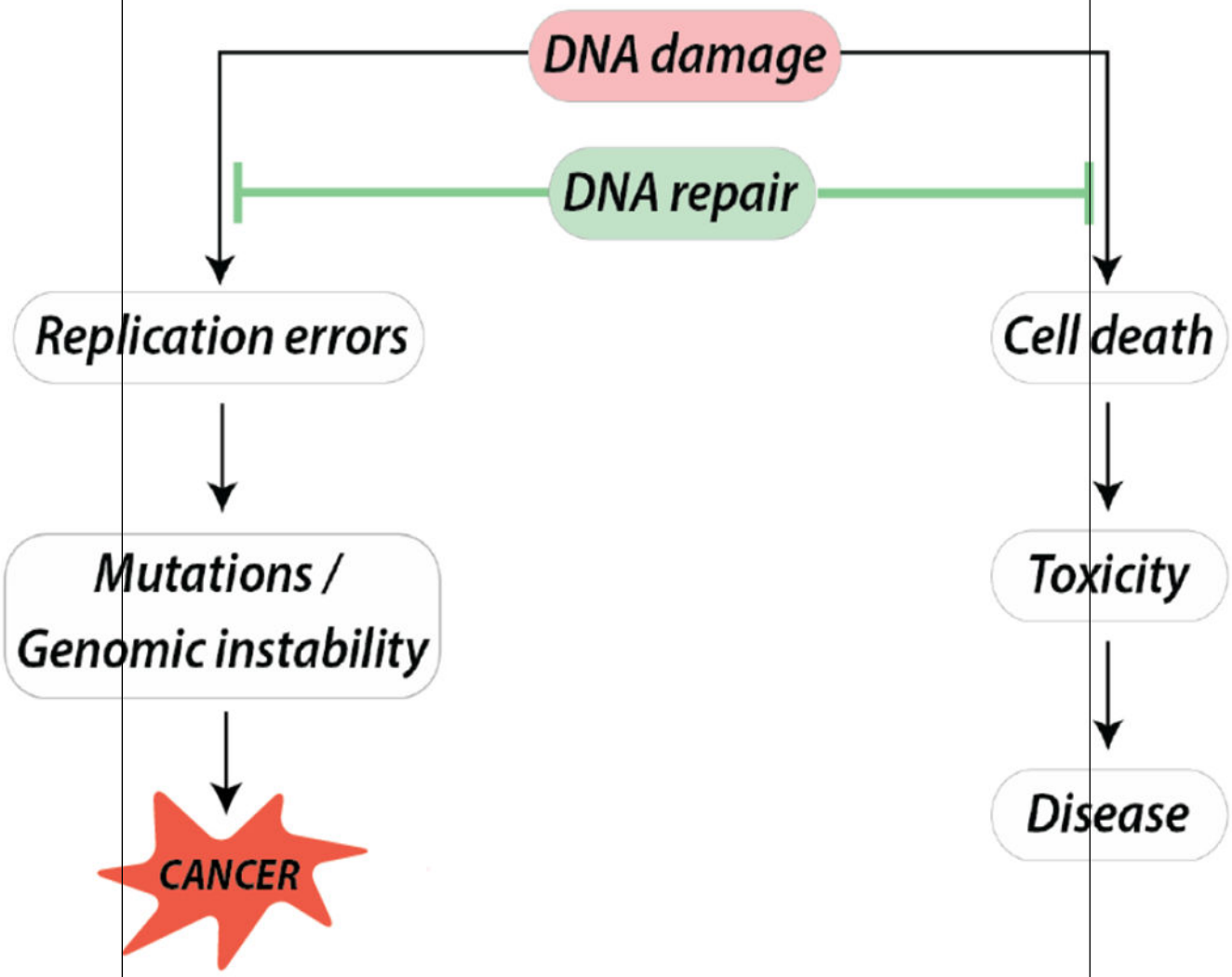

Figure I: DNA damage response pathway.

DNA damage can cause replication errors that can lead to an accumulation of mutations that promote carcinogenesis. Inefficient repair can also promote DNA damage-induced cell death, leading to increased toxicity and disease

DNA damage plays a major role in cancer initiation and progression. Incorrect or inefficient repair of damaged DNA can lead to genetic alterations that facilitate tumor formation (Figure I). More specifically, DNA damage-driven losses, amplifications or other alterations in the genome can promote cancer by affecting expression of important tumor suppressor genes and oncogenes. It is therefore unsurprising that deficiencies in DNA repair are associated with an increased risk for cancer, in some cases having dramatic effects [70]. Even among apparently healthy individuals, more subtle differences in DNA repair capacity can modulate cancer risk [71].Ironically, inflicting

DNA damage is the most common approach for treating cancer, since at high enough levels, DNA damage kills tumor cells. These facts point to utility in being able to measure DNA damage, DNA repair, mutations and cytotoxicity.

Past and emerging breakthroughs in high-throughput technologies are enabling us to visualize and quantify DNA damage formation and its clearance by DNA repair. These technical advances promise improvements in precision treatment of cancer, since they can be used reveal both the extent of DNA damage inflicted on tumors cells, and the ability of those cells to clear away the damage. Biomarkers of DNA repair capacity thus promise utility in personalized cancer treatment, as well as in grouping patient subpopulations according to the likelihood of responsiveness of their tumors to novel chemotherapeutics. Existing and emerging methods of measuring DNA repair capacity are also being used for cancer epidemiology, since they are making it possible to group populations according to their DNA repair capacity and then query the relationship between repair capacity and cancer susceptibility. Finally, the ability to quantify DNA damage can be useful in cancer prevention, since DNA damage would indicate whether certain environmental chemicals are potential carcinogens, and quantifying DNA repair capacity would identify at-risk individuals who would then have the possibility of avoiding key exposures. Taken together, being able to measure DNA damage levels and DNA repair capacity has applicability in precision medicine, patient stratification, epidemiology, cancer prevention and precision prevention.

Highlights:

- Functional cell-based assays provide an integrated view of all of the factors that affect efficiency of repair pathways, including single nucleotide polymorphisms, expression level, protein stability, and post translational modifications.
- Fluorescent cell-based assays can be useful as a tool to screen chemicals for their DNA damaging potential and for their impact on cell growth, which has applications in public health.
- Tools to quantify DNA damage and repair in people have utility in personalized medicine and precision prevention.

Outstanding Questions:

- Can repair trapping be used as a way to reveal inter-individual difference in repair capacity for DNA adducts?
- To what extent does DNA repair capacity vary among cell types?
- Does repair efficiency predict cancer susceptibility, and is predictive power constrained to certain types of cancers?
- Are there as yet unknown avenues for leveraging knowledge about DNA repair capacity to optimize a person's cancer treatment regimen?
- How well does the frequency of homologous recombination events predict downstream cancer?

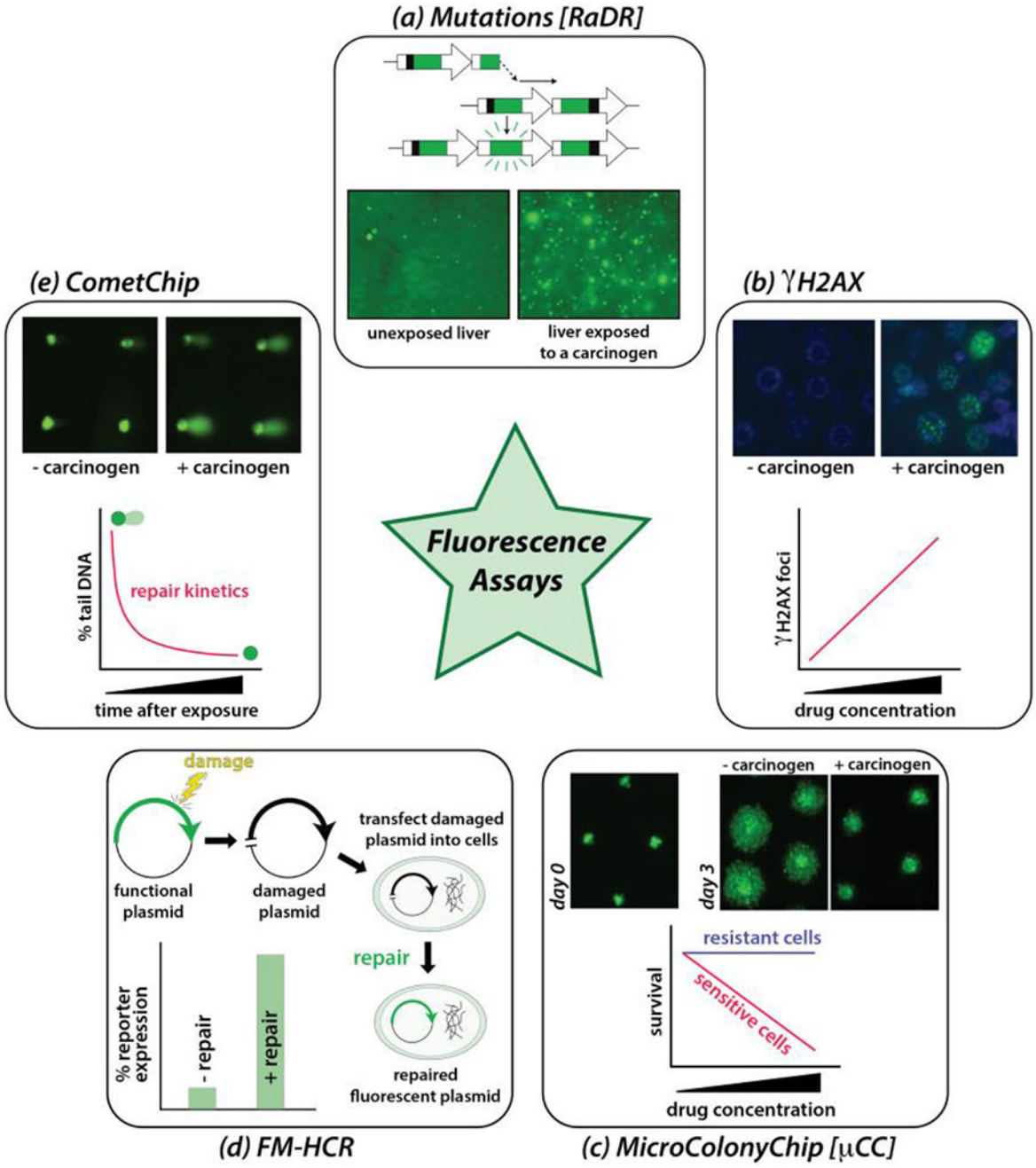

Figure 1:

Live-cell fluorescent based assays are enabling us to visualize and quantify DNA damage. (a) RaDR assay for visualizing mutations *in vivo* (b) γH2AX for analysis of DNA repair foci that are recruited at sites of DSBs (c) MicroColonyChip to analyze cell cytotoxicity by measuring the ability of cells to divide and form colonies in the presence of damaging DNA damaging agents (d) FM-HCR measures ability of cells to repair plasmid reporters carrying specific types of damage (e) CometChip assay measures DNA strand breaks.