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Immunofluorescence Imaging of DNA Damage Response

Proteins:

Optimizing Protocols for Super-resolution Microscopy

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Abstract

Immunofluorescence imaging has provided captivating visual evidence for numerous cellular events, from vesicular trafficking, organelle maturation and cell division to nuclear processes including the appearance of various proteins and chromatin components in distinct foci in response to DNA damaging agents. With the advent of new super-resolution microscope technologies such as 4Pi microscopy, standard immunofluorescence protocols deserve some reevaluation in order to take full advantage of these new technological accomplishments. Here we describe several methodological considerations that will help overcome some of the limitations that may result from the use of currently applied procedures, with particular attention paid to the analysis of possible colocalization of fluorescent signals. We conclude with an example of how application of optimized methods led to a breakthrough in super-resolution imaging of nuclear events occurring in response to DNA damage.

Keywords

immunofluorescence; antibody purification; RNAi to assess antibody specificity; antibody mixes; antibody cocktails; sequential antibody staining; super-resolution microscopy; 4Pi microscopy

1. Introduction

The mammalian DNA damage response is a complex signaling cascade involving the coordinated physical and functional interaction of a large body of proteins that are generally grouped into three interacting pathways: (i) DNA damage sensing, (ii) transduction and amplification of the damage sensing signal and, (iii) downstream effector activities that carry out end point functions such as DNA replication fork stalling, chromatin remodeling

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and DNA repair. The complicated yet rapid and efficient action of these pathways is necessitated by a crucial overall goal - the maintenance of genome integrity in the face of continuous insult from both internal metabolic processes and exposure to external mutagens. This view is strongly supported by the fact that defects in many of these activities are associated with increased risk of cancer (1–3), and these findings have shaped the concept of the DNA damage response as a barrier to oncogenic transformation (2, 3).

Multiple genetic, biochemical and now proteomic approaches have provided a vast database of information regarding the functions, post-translational modifications, e.g. phosphorylation and ubiquitination, and associations of many protein and chromatin components of these pathways in response to DNA damage (4–6). Immunofluorescence studies have played a leading role in providing significant advances to our current cellular view of the spatial and temporal dynamics of these components, and it is this aspect of the methodological repertoire that is the focus of this review.

Some of the earliest studies of DNA damage repair proteins that included immunofluorescence images suggested great promise for this technique in revealing visual details of the damage signaling and repair processes. The eukaryotic Dmc1 and Rad51 recombinase proteins were seen to form nuclear foci associated with chromosomes during meiosis or following exposure of cells to DNA damage (7–9), results suggesting that these foci represented active regions of recombination. A functional ATM kinase, an immediate responder to DNA damage (10–12), was shown to be required for formation of human Mre11 and Rad50 nuclear foci, suggesting a functional interplay between these proteins at the very early stages of the DNA damage response (13). Thus, the groundwork was established for further immunofluorescence efforts to analyze all features of the DNA damage response, including histone modifications, cellular and nuclear relocalization of proteins, formation of foci and various protein associations, as well as protein activation, e.g. using phospho-specific antibodies to detect activated ATM. At this point, the use of immunofluorescence methods has created an intriguing and seemingly well-choreographed set of events describing many aspects of the DNA damage response (12, 14, 15).

However, there remain significant methodological barriers that have precluded bringing appropriate clarity to the images and molecular models derived from immunofluorescence studies. The primary objective in our review is the presentation of several methods relevant to the acquisition of target-specific, optically resolved immuno labeled images of DNA damage signaling and repair proteins in mammalian cells. Given the diversity of image acquisition tools and analyses available, combined with the unprecedented speed at which new sub-diffraction resolution microscopes are being introduced, commonly applied methods require some reexamination and retooling in order to take advantage of the improved imaging capabilities now available. It is vitally important that samples are prepared, stained and evaluated in a manner that does not proliferate artifact, both in collection and analysis. To that end, important features of this review are descriptions of methodological caveats that the user should consider before engaging in the collection of microscopy data. These include assessment of cell growth, fixation, and blocking methods, as well as antibody host, quality and specificity. We specifically address concerns regarding the application of multiple antibodies to fixed cells for the purpose of determining possible protein colocalization. While our descriptions focus on proteins involved in the DNA damage signaling and recombinational DNA repair pathways in human cells, the methods described here will be generally applicable to most immunofluorescence studies.

2. Antibody quality and specificity

2.1. Antibody purification

Clearly, the usefulness of an antibody for immunofluorescence protocols requires a far more stringent assessment of its specificity than for certain other techniques. While irrelevant cross-reacting proteins can often be ignored in Western blotting procedures, assuming that the cross-reacting proteins migrate at distinctly different positions on the SDS gel than the protein of interest, no such cross-reacting material can be tolerated in immunofluorescence images. Many procedures have been developed for the purification of IgG molecules from raw serum samples or mouse ascites fluid that involve affinity purifications or the use of IgG-specific resins containing coupled *Staphylococcus* protein A or *Streptococcal* protein G (16, 17), and numerous protocols exist on various company websites. Many protocols involve elution from the affinity resin using low pH buffers, which can severely reduce antibody quality if the time of exposure is too long. However, we have found that a modified protein A (Bio-Rad) allows efficient and quantitative elution of IgG from both serum and ascites fluid using buffers with a pH ranging from 4.5 – 6.5. Additionally, to minimize possible damage to the antibody when using buffers at the lower end of this range, we elute directly into a Tris-based buffer at pH 9.0 resulting in a product that is either at or near physiological pH. For purifications starting with larger volumes of ascites fluid or serum, we always divide the samples such that multiple 1 – 2 ml bed volume columns are used as opposed to larger volume columns, thus reducing the time of exposure of protein to the lower pH. Finally, we find it critical to buffer-exchange and store the purified product in phosphate buffered saline (PBS) to ensure that it is maintained at physiological pH. The Western blot in Figure 1 shows an example of how purification of a commercially available monoclonal antibody directed against the human Xrcc3 protein (originally obtained from Novus) can offer a significant improvement in antibody specificity. Whereas multiple cross-reacting bands appear when using the raw ascites fluid containing the anti-Xrcc3 antibody, use of the modified protein A purified IgG results in the appearance of a single protein band at the appropriate molecular weight, 37.8 kDa.

For immunofluorescence studies, control experiments demonstrating the exclusive interaction of an antibody with its specific antigen are an absolute must because there is no reliable way for the researcher to distinguish specific from non-specific signals in stained cells. Thus, immunofluorescence work should include the use of purified and well-characterized antibodies. As an example of the problems that can result when using non-purified antibodies, even those that may show only a trace of cross-reactivity, the images in Figure 2 compare the use of a non-purified and purified anti-Xrcc3 antibody. HEK293 cells were methanol-fixed (–20 °C, 8 min) 2 hr following exposure to 8 Gy ionizing radiation (IR), a treatment frequently used to induce DNA damage including DNA double strand breaks (DSB). Cells were stained for Xrcc3 using either the raw ascites fluid or the purified antibody. With the raw ascites fluid signal is observed throughout the entire volume of the cell in a diffuse, seemingly non-specific manner. In contrast, use of the purified anti-Xrcc3 antibody produces signals referred to as nuclear foci, which are characteristic of many DNA damage signaling and repair proteins. In this case, foci are also observed in the cytoplasm and Xrcc3 also appears to aggregate near the perinuclear region. While numerous reports suggest that nuclear foci correlate with sites of protein activity, the functional relevance of protein foci remains controversial. Here however, our concern is with specific caveats regarding immunofluorescent protocols and controls that aid in proper interpretation of images. Using the example of human Xrcc3, in addition to human Rad51 and Rad51C, in the next section we describe a critical set of controls that we have included previously (18) and suggest be included in all immunofluorescence studies to ensure proper interpretation of images.

2.2. Assessing the quality, specificity and usefulness of antibodies for immunofluorescence protocols

In the DNA damage signaling and repair literature, controls are sometimes included showing antibody specificity using Western blot analyses of extracts from cells depleted for the target protein, either genetically or using RNAi methods. However, there are far fewer reports showing loss of immunofluorescent signals using these methods. In fact, the complete or near complete loss of immunofluorescence upon depletion of the target is one of the best tests of antibody specificity, as this demonstrates the lack of any significant cross-reacting material that might interfere with interpretation of the image. Figure 3 provides examples of this type of analysis. HEK293 cells were transfected with small interfering RNA duplexes (siRNA; SMARTpool, Dharmacon) specific for human Rad51, Rad51C or Xrcc3 (control cells were treated only with the lipid transfection reagent, Dharmafect) and grown for 48 hr. At 2 hr following exposure to 8 Gy IR, cells were fixed and stained with modified protein A purified antibodies against human Rad51, Rad51C or Xrcc3 followed by incubation with a highly cross-absorbed goat anti-mouse Alexa 488 secondary antibody (Molecular Probes). While control cells show characteristic nuclear foci for all three proteins, as well as cytoplasmic and perinuclear staining (Figure 3A, B and C), cells treated with specific siRNAs showed nearly complete loss of signal (Figure 3D, E and F; microscope settings were identical for all images A–F). The minor amount of residual signal in some images, e.g. Rad51 and Xrcc3, is most likely due to a small background contributed by the Alexa 488 secondary antibody (Figure 3G and H). RNAi-mediated knockdown of proteins was also confirmed by Western blots (not shown). Therefore, interpretations regarding protein sub-cellular distribution and alterations in this distribution in response to DNA damage can be made with much more certainty if controls such as these are used to demonstrate antibody specificity. Given the complexity of the interactions, functions and cellular distribution of the large number of proteins involved in DNA damage sensing, signaling and repair, it is critically important that the reagents and methods used conform to exceptionally high standards. Additionally, given the vast differences in antibody quality available from commercial sources, these types of controls should be considered an important inclusion in all published immunofluorescent studies.

3. Cell preparation and staining optimization

In this section we discuss several considerations of cell growth, fixation and staining methods that are of great significance for producing optimal immunofluorescent images.

3.1. Importance of cover slip thickness in high-performance optical microscopy

In preparation for immunofluorescent staining, cells are typically grown on cover slips, which can be seen as the first “lens” of a microscope objective lens. Its quality is, therefore, essential for high-quality microscopy. The most important feature of the cover slip is its thickness, since the design of a high-performance objective lens assumes a certain cover slip thickness. Most objectives have a range of cover slip thickness for which they are optimized printed on their collar. If the thickness is outside this range spherical aberrations are introduced into the imaging path, leading to potentially severe degradation of image quality. The z-resolution is especially affected by these aberrations.

Most high-quality objectives are optimized for a 0.17 mm cover slip thickness. This is especially important for objectives with high numerical apertures (NA), which provide the highest resolution, and objectives optimized for water immersion or dry usage, due to the large refractive index mismatch compared to the glass. Objectives with correction collars often allow adjustment for different cover slip thicknesses (1.2 NA water immersion Zeiss

40x, 63x, Leica 63x: 0.14 – 0.18 mm). 0.17 mm thickness is, therefore, suited for the largest variety of high-NA objectives (with or without a correction collar).

The industry standard, #1.5 (equivalent to 0.16 – 0.19 mm), is typically the best match. However, in practice many manufactures deliver cover slips with thicknesses at the upper edge of the specified range. For critical applications the thickness of the cover slips should, therefore, be checked with a micrometer. Alternatively, more tightly specified cover slips are available at higher prices.

3.2. Fixation methods

A variety of cell fixatives and permeabilization agents are available, with the most commonly used being formaldehyde/paraformaldehyde (PFA) and methanol (19, 20). In the DNA damage signaling and repair literature the most frequently used fixation protocols are (i) 1–4% PFA with 0.1–0.5% Triton X-100, (ii) 1% formaldehyde on its own, (iii) a 1:1 mixture of methanol:acetone, and (iv) methanol on its own at –20 °C. We have found very similar results in most of our studies using 4% PFA with 0.1% Triton X-100 for 10 min, or –20 °C methanol on its own for 8–10 min. Both protocols suffice to fix and permeabilize cells with no apparent distortion of cellular ultrastructure and protein positioning as judged by separate stainings for actin, α - and β -tubulin, nuclear pore and nucleoplasmic proteins, and chromatin components (not shown). Certain fluorophores are quenched to varying degrees by PFA or methanol, and control studies should be included to assess this. We have used –20 °C methanol for the work presented here and in most of our studies (18, 21). For studies using high resolution 4Pi microscopy [see section 4 below and (22)] we include an important additional fixation step of 4% PFA for 5 min following staining with primary and secondary antibodies in order to both fix the position of the antigen-antibody complexes which prevents some time-dependent dissociation, as well as to prevent the positional fluctuation (restricted Brownian motion) of the secondary fluorophore conjugates that can actually be observed at the resolution provided by 4Pi microscopy.

3.3. Blocking agents

To reduce background fluorescence, sample preparation typically includes a blocking step. Most frequently the blocking buffer is PBS with one of the following agents: (i) 1–5% BSA IV; (ii) 2–5% non-fat dry milk; (iii) 5–10% goat serum plus 0.1% NP-40; or (iv) 10% fetal calf serum. When blocking with serum, the general rule is to use a normal serum from a species different from that used to generate the primary or secondary antibodies. Despite the normal precautions, we still observe varying levels of background when using certain traditional blocking agents. However, we find that background can be virtually eliminated with a marine blocking agent because it has essentially no antigens that can cross-react with secondary antibodies derived from commonly used mammalian species, e.g. rabbit, mouse, donkey or goat. In Figure 4 we compare the background fluorescence when fixed HeLa cells are blocked with a marine agent (MAXblock™, Active Motif) (Fig. 4A), 5% non-fat dry milk (Fig. 4B) or 5% BSA IV (Fig. 4C), followed by incubation with an anti-rabbit secondary Alexa 647 conjugate at a 1:250 dilution. This dilution is less than what is typically used, e.g. 1:1,000 – 1:2,000, to exaggerate the potential appearance of non-specific background signal. Clearly, the marine blocking agent virtually eliminates any background staining whereas the non-fat dry milk and BSA show background that would interfere with interpretation of the image. Marine blocking agents are very cost effective compared to BSA IV or various sera. They currently serve as the best blocking agent for studies of mammalian cells, and are found with increasing frequency in the immunofluorescence literature.

3.4 Double staining methods to assess proximity or colocalization

Colocalization analysis is frequently used to discern the spatial relationship of the numerous proteins and chromatin components that form visible clusters following exposure of cells to genomic insults. However, many factors, including sample preparation, use of uncharacterized antibodies, fluorescent cross-talk that can result from the use of inappropriate image acquisition parameters, filter sets and image processing programs can easily contribute to the appearance of colocalization. These and other caveats, as well as useful guides for fluorescence colocalization analysis are described in several reviews (23–28). Alison North also presents an excellent review in which she describes colocalization as “the most common example” of misleading information derived from immunofluorescence studies where appropriate consideration of these many factors is not included (29). Further discussion of colocalization analysis relevant to super-resolution microscopy is presented below in section 4. Here, we present an experimental consideration that is particularly relevant to the field of DNA damage signaling and repair. To briefly summarize the following description, the use of antibody mixes or cocktails containing IgGs from different species can enhance or create the appearance of colocalization if one of the epitopes is in significant excess over the other.

It has long been assumed that the use of antibody mixes or cocktails, with each antibody directed against a different target, is acceptable as long as the antibodies derive from different species, e.g. a rabbit polyclonal and a mouse monoclonal. In experiments shown here we find a dramatically different outcome regarding colocalization of the human Rad51 protein and γ H2AX when using an antibody cocktail staining procedure vs. a sequential staining protocol. γ H2AX is a phosphorylated version of the histone variant H2AX that in mammalian nuclei appears very rapidly following exposure of cells to DNA damage. γ H2AX has been widely used as a marker for DNA double strand breaks following its discovery by the Bonner group in 1998 (30). In numerous studies proteins are shown to colocalize with γ H2AX and from methods descriptions it appears that mixtures of primary antibodies, and subsequently secondary antibodies, are used for immunostaining (14, 15, 31–38). In Figure 5, we compare two immunostaining protocols, one using antibody cocktails and another in which antibodies are applied sequentially. Primary antibodies were a purified rabbit polyclonal against human Rad51 (Novus) and a purified mouse monoclonal against γ H2AX (Upstate – now Millipore). Secondary antibodies were either highly cross-adsorbed donkey anti-rabbit Alexa 488 and donkey anti-mouse Alexa 647 conjugates, or the same with goat as the host ensuring that there is no host secondary effect. HEK293 cells were exposed to 8 Gy IR, cultured for 2 hr, fixed in methanol (–20 °C, 8 min) and blocked overnight using a marine blocking agent (MAXblock™, Active Motif). Using the antibody cocktail protocol, fixed cells were incubated with a mixture containing the anti-Rad51 and anti- γ H2AX antibodies, each at a 1:1,000 dilution, for 1 hr at 37 °C. Cells were washed in PBS (5 changes for 5 min each), then incubated with a cocktail containing the anti-rabbit and anti-mouse secondary antibodies, each at a 1:1,000 dilution, for 1 hr at 37 °C and washed again. Coverslips were mounted using Vectashield with or without DAPI, sealed in polyurethane and stored at 4 °C in the dark. Using the sequential staining protocol, fixed cells were stained first with the rabbit anti-Rad51 antibody followed by the anti-rabbit secondary, then the anti- γ H2AX antibody followed by the anti-mouse secondary. All antibody dilutions, washes and coverslip mounting were done as above. Cells were visualized using a Leica TCS SP2 AOBS confocal microscope and image processing performed using the accompanying Leica software. Microscope settings were identical for all images taken in both staining protocols. Using antibody cocktails, Rad51 and γ H2AX appear exclusively in the nuclei (Fig. 5A and 5B). The merge image and the accompanying cytofluorogram (Fig. 5C and 5D) show nearly perfect coincidence of the fluorescent signals. Using the sequential staining protocol, we find that while γ H2AX appears exclusively in the

nuclei as expected (Fig. 5F), Rad51 shows both distinct nuclear focal staining, as well as a robust cytoplasmic signal (Fig. 5E). Additionally, the merge image and accompanying cytofluorogram (Fig. 5G and 5H) show little coincidence of the nuclear Rad51 and γ H2AX signals. These same differences between the cocktail vs. sequential protocols were observed using different primary and secondary antibodies, in this case a purified mouse monoclonal against Rad51 and a purified rabbit polyclonal against γ H2AX (not shown).

It is also worth noting that the 488 and 647 nm fluorophores have no spectral overlap, which eliminates the possibility of bleed-through. Additionally, these were sequential scans requiring that only one laser line be active at a time, which again eliminates any possible bleed from channel to channel.

Is it reasonable to expect such a robust Rad51 cytoplasmic signal? In fact, there are many accounts in the literature showing significant amounts of cytoplasmic Rad51 in various mammalian cell lines (18, 21, 35, 39–46), and in some cases cytoplasmic Rad51 is clearly apparent by immunofluorescence (18, 21, 41, 42). Further, we show here (Figure 3) and in published work (18) that the fluorescence signal for Rad51, as well as Rad51C and Xrcc3, is eliminated when cells are depleted of the protein using specific siRNAs, thus strongly supporting the veracity of the observed cellular localization.

How then might we explain the significant difference in fluorescent signal appearance using the cocktail vs. sequential staining protocols? We performed several experiments to ask whether the staining of γ H2AX somehow influences that for Rad51, reasoning that the possible extreme excess of γ H2AX antigen following DNA damage relative to Rad51 may negatively influence Rad51 staining. Using primary antibodies against γ H2AX and Rad51 that were both rabbit polyclonals, we performed a sequential staining procedure in which cells were incubated first with the anti-Rad51 polyclonal, washed extensively, incubated with the anti- γ H2AX polyclonal, washed extensively again and incubated with an anti-rabbit Alexa 488 secondary. We expected to see an image similar to the merge in Figure 5G indicative of nuclear localization of γ H2AX, and both nuclear and cytoplasmic localization of Rad51. Surprisingly, we found only nuclear staining (Fig. 6A and B). Identical images were seen using mouse monoclonals against both proteins followed by incubation with an anti-mouse Alexa 647 secondary (not shown). Therefore, given the opportunity to interact with both primary antibodies, the secondary appears to see only the anti- γ H2AX primary antibody. These results suggest that the primary γ H2AX antibody out-competes the primary Rad51 antibody for interaction with the secondary antibody. This is most likely explained by the excess epitope availability of γ H2AX relative to Rad51 following DNA damage. Therefore, it stands to reason that we could recover the appropriate Rad51 signal by reducing the amount of γ H2AX primary antibody used in the staining procedure. The images in Figures 6C and D show the results of such an experiment. In this case cells were stained using our standard sequential staining procedure and a 1:1,000 dilution of anti-Rad51 rabbit primary antibody, but the anti- γ H2AX rabbit primary antibody was used at a 1:10,000 dilution. Subsequent staining with the anti-rabbit Alex 488 secondary reveals fluorescent signal in both the nucleus and cytoplasm. This result suggests that the amount of γ H2AX epitope available following the response of cells to DNA damage creates a situation where there is such an excess of primary γ H2AX antibody that it out-competes the Rad51 primary for interaction with the secondary antibody.

These data strongly support the idea that relative epitope abundance can contribute to the misleading appearance of colocalization if antibody cocktails are used, particularly for experiments involving high abundance epitopes such as γ H2AX following DNA damage. Interestingly, if the use of antibody cocktails had shown excessive cytoplasmic staining, a methodological red flag would have been raised. Unfortunately, the exclusive nuclear

staining of this pair is easily rationalized in terms of DNA damage and repair events, and this particular procedural caveat went unnoticed. While there is very likely some degree of fluorescent signal coincidence between γ H2AX and various other proteins, the degree to which this effect is enhanced by the use of antibody cocktails deserves to be reconsidered. As noted above, a number of other factors that have been ruled out in the experiments described here can additionally contribute to this problem. We also note that non-specific aggregation of IgG molecules occurs as concentrations increase, such as in a cocktail, and this may also contribute to the appearance of colocalization. Therefore, if antibody cocktails are used, it is very important to provide controls demonstrating that each antibody behaves similarly when used either individually or in a cocktail. While it may seem that the multiple steps used in the sequential staining protocol are excessively time consuming, use of this procedure avoids the possible misleading appearance of inappropriate colocalization or the enhancement of an otherwise limiting amount of signal coincidence.

4. Super-resolution microscopy exemplified by 4Pi

While proper sample preparation and imaging protocols as described above avoid common colocalization and other imaging artifacts, we want to point out that the observation of structures and determination of colocalization is limited by the resolution provided in the recorded images. Distinguishing between tightly associated target molecules on the one hand and molecules separated by as much as the resolution limit of the microscope, typically one hundred times the diameter of a typical protein, on the other hand is difficult. Sophisticated methods such as Förster Resonance Energy Transfer (FRET) take advantage of photo-physical phenomena like dipole-dipole interactions between neighboring fluorescent probes to measure distances on the length scale of a single protein (a few nanometers), but not larger. Its application is not suitable to resolve complex structures and requires careful experimental design and data analysis to avoid artifacts. It is, therefore, beyond the scope of this review and we refer the reader to two of many excellent reviews (47, 48). Here we want to focus on conventional structural and colocalization analysis, its limitations and how super-resolution microscopy, especially 4Pi microscopy, can provide new information.

Image resolution is determined by (i) the pixel size, which can be easily controlled by choosing a suitable magnification of the microscope, and (ii) optical resolution. Choosing a pixel size approximating half the optical resolution value renders it non-limiting and is, therefore, highly recommended. The optical resolution, however, is fundamentally limited by diffraction and cannot be adapted easily. Even the best objectives with the highest NAs available provide only about 200 nm resolution in the focal plane. The depth (or axial) resolution is at least 2.5 times worse, making it impossible to differentiate between two objects that are axially separated by 500 nm or less. Using a regular wide-field microscope, axial discrimination, especially of bulky objects within a DAPI-stained nucleus, becomes extremely difficult or even impossible due to the strong out-of-focus background contribution to the signal. In this case, colocalization studies are often reduced to analyzing two-dimensional (2D) images generated from single recordings or projections of three-dimensional (3D) stacks along the axial direction. This reduces the 3D nuclear architecture to a 2D view and, therefore, weakens statements based on resulting colocalization studies. Especially for objects present at high density it is difficult to decide whether two objects really coincide in all three dimensions or are located at different depths of the sample. Confocal laser scanning microscopy provides true 3D resolution by suppressing out-of-focus light. For colocalization studies of inherently 3D objects, confocal microscopy or alternative true 3D-imaging techniques are, therefore, recommended.

To observe fine structures at the 100 nm level or to narrow colocalization information down to these values or less, super-resolution microscopy techniques are necessary. Recent years have seen the emergence of several alternative techniques such as STED, RESOLFT, FPALM, PALM, STORM and others summarized by Stefan Hell in a recent review (49). Here we concentrate on 4Pi microscopy, the first one of these techniques to become commercially available and provide 3D imaging capabilities suitable for the analysis of nuclear structures.

4Pi microscopy is a laser scanning microscopy technique that enhances the axial resolution of standard confocal microscopy 5 – 7 fold. This is achieved by doubling the solid aperture angle used for excitation and/or detection using two opposing objectives that focus into the same spot in an optically coherent arrangement. In the favored Type C variant of 4Pi microscopy the excitation wavefronts passing through the two objectives interfere constructively, and the focus center-emitted light is collected by both objectives and recombined constructively. This results in a sharp central focus of 100 nm axial width (50). Side maxima, which additionally appear, are removed by processing the recorded data with an image restoration (deconvolution) algorithm. Imaging the cell nucleus with 4Pi microscopy, as well as with most other super-resolution microscopy techniques, requires special care due to the strong refractive index mismatch between the nucleus and typical aqueous embedding media such as PBS. Matching the refractive index of the embedding medium with the nucleus by using glycerol at a high concentration (80 – 90%) and using glycerol immersion lenses (100x/1.35 NA) (22), or alternatively 2, 2'-thiodiethanol and oil immersion lenses (100x/1.46NA) (51) provides conditions suitable for super-resolution imaging of the nucleus.

In Figure 7 we show a single optical section taken from a 3D data set of a HeLa cell nucleus stained for histone H2AX, comparing standard confocal microscopy (Fig. 7A) to 4Pi microscopy (Fig. 7B). The clusters of H2AX observed are much more clearly resolved in the 4Pi data set and allow for automatic differentiation and quantification (22). The superior resolution offered by 4Pi allowed us, for example, to determine that the diameters of H2AX clusters are 100 – 600 nm less than the much larger diameters of γ H2AX clusters seen following exposure of cells to IR (22).

Colocalization can be quantified best by analysis using multidimensional histograms, or “cytofluorograms”, where each axis represents one color-channel. Every pixel (or voxel) of a 2D (or 3D) data set is added to this histogram according to its values in each of the analyzed color channels. High levels of colocalization lead to an accumulation of pixels along the diagonal of the histogram while non colocalized staining shows up as signal along the axes, for example see Figure 8B. A problem often occurring in fluorescence microscopy is the large fraction of dark background pixels. They are represented close to the origin of the histogram as a very large peak with an amplitude that can easily exceed the values representing brighter structures by a factor of 1,000 or more. To better visualize the more interesting fraction of pixels representing brighter cellular structures, we recommend using a logarithmic color table for the amplitudes in the histogram or cutting off the histogram amplitudes at lower levels. Colocalization can be quantified by comparing the integrated amplitudes in different regions of interest (ROIs) in the histogram. Care must be exercised in defining the ROIs so as not to include the large amplitudes of the background peak close to the origin. This method is preferred over conclusions from purely visual interpretation of the recorded images. Judging by eye often underestimates the contribution of dimmer structures in the sample. An additional problem arises in 3D representations of the data due to the fact that most 3D visualization packages do not provide good ways to represent voxels with two colors present simultaneously. In these cases most software packages will demonstrate a preference for one color over the other, hiding the other one or resulting in camouflage-like

patterns that are not representative of the protein distribution. This effect as well as limitations in quantitative data analysis tools, which often are not fully implemented for 3D data stacks, limit the use of commercially available software and often require custom-made programs or macros for programs such as ImageJ.

Colocalization studies depend critically on the achieved resolution in the analyzed image data. 4Pi microscopy, for example, enabled us to clearly distinguish neighboring clusters of H2AX and γ H2AX at 100 nm resolution. This is seen in Figure 8, in which signals of neighboring clusters are spatially separated (Fig. 8A) and a cytofluorogram of the 4Pi 3D data sets (Fig. 8B) reveals that, in fact, H2AX and γ H2AX clusters do *not* colocalize (22).

While 4Pi microscopy provides a 5 to 7-fold improvement in 3D resolution over standard confocal microscopy, the achieved 100 nm resolution is still more than a factor of 10 larger than the diameter of a typical protein. Some of the more recent super-resolution microscopy techniques, namely STED and the FPALM/PALM/STORM techniques, have reached 20 to 30 nm resolution over the last years (49). In the DNA damage and repair field, the 3D imaging variants of these instruments are of particular interest because they provide the means to explore complex structures in the nucleus at nearly molecular resolution. The year 2008 has seen the emergence of 3D-STORM (52), 3D-FPALM (53) and isoSTED (54), which provide 20–75 nm resolution in all three directions simultaneously. Multi-color and live cell imaging is also being developed at a fast pace (55–60). The resolution of these new microscopes approaches the size of the fluorescent labels used, now posing a new limitation, which only a few years ago was still far out of reach. While this problem is being tackled, however, the time is ripe to apply these techniques already at the “moderate” resolution of 20 – 75 nm to studies of DNA damage signaling and repair, thus providing new visual insights into these complex processes at resolving volumes approximately 1,000-fold smaller than achieved by conventional confocal microscopes.

5. Concluding Remarks

Immunofluorescence studies of DNA damage signaling and repair proteins have provided a wealth of information regarding the cellular distribution, response to DNA damage and potential association of the many proteins and chromatin components that play important roles in these pathways. As the field now embraces the use of extraordinarily high resolution optical methods, the stringency of all aspects of cell preparation, staining and image analysis must be well controlled in order to fully realize the advantages of the increased resolution offered by these new technologies. It is especially important for researchers to ensure that antibody preparations and application to fixed samples are optimized such that resulting images accurately reflect the biological complexities of these intricate and networked pathways.

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Figure 1. Purification of anti-HsXrcc3 monoclonal antibody enhances specificity in Western blot procedures

HEK293 whole cell extracts were analyzed by Western blot for the presence of the Xrcc3 protein. Neighboring lanes of a 10% SDS polyacrylamide gel were loaded with 60 μ g total protein, and following transfer to a nitrocellulose membrane the lanes were separated. One was incubated with ascites fluid containing anti-HsXrcc antibody and the other was incubated with antibody purified from the ascites fluid. Incubation times with antibody, washes, incubation times with VisualizerTM ECL reagent (Upstate, Inc. – now Millipore) and exposure times were identical for both blots.

Figure 2. Purification of anti-HsXrcc3 monoclonal antibody enhances specificity and detection of protein by immunofluorescence

HEK293 cells exposed to 8 Gy IR were fixed and stained with either anti-HsXrcc3 mouse ascites fluid (left) or purified anti-HsXrcc3 antibody (right), followed by staining with a goat anti-mouse Alexa 488 secondary antibody conjugate. Antibody dilutions were 1:500 (primary) and 1:1,000 (secondary).

Figure 3. Antibody specificity as determined by RNAi-mediated depletion of target protein
 HEK293 cells were mock transfected (A, B, C) or transfected with the indicated siRNA SMARTpools, Dharmacon (D, E, F). At 48 hr post-transfection cells were exposed to 8 Gy IR, grown for 2 hr and fixed and stained with the indicated purified monoclonal primary antibody followed by staining with a goat anti-mouse Alexa 488 secondary antibody conjugate. Antibody dilutions were 1:1,000 (primary) and 1:1,000 (secondary). DNA was counterstained with Vectashield containing DAPI. Panels G and H show non-transfected HEK293 cells exposed to 8 Gy IR as above but stained only using the goat anti-mouse secondary Alexa 488 secondary conjugate. Images were obtained using a Leica SP2 confocal system. Image acquisition parameters were identical for each collection.

Figure 4. A marine blocking agent (MAXblock™) improves staining quality relative to standard blocking agents

HeLa cells were fixed with methanol (−20 °C, 8 min) and blocked overnight in PBS containing (A) MAXblock™, Active Motif, (B) 5% non-fat dry milk or (C) 5% BSA. Cells were washed and staining only with an anti-rabbit secondary Alexa 647 conjugate using a 1:250 dilution. Images were collected using a Zeiss Observer Z.1 in widefield mode and exposure times were the same for each collection. These images appear on the Active Motif web site and are used here with permission from Active Motif <http://www.activemotif.com>. MAXblock™ was developed by Dr. Brian Bennett.

Figure 5. Use of antibody cocktails can lead to misleading appearance of co-localization
 HEK293 cells were exposed to 8 Gy IR, fixed and stained using either a cocktail containing a Rad51 polyclonal and γ H2AX monoclonal antibody (A–D), or using a sequential method in which the Rad51 polyclonal antibody was applied first followed by the anti-rabbit secondary, and then the γ H2AX monoclonal antibody followed by the anti-mouse secondary (E–H; see text for detailed description of methods).

Figure 6. Reduction in the concentration of γ H2AX primary antibody reduces competition with Rad51 primary for binding of the secondary antibody

HEK293 cells were exposed to 8 Gy IR, fixed and stained sequentially, first using the Rad51 polyclonal and then the γ H2AX polyclonal followed by washing and staining with an anti-rabbit Alexa 488 secondary conjugate. Dilutions of primary antibodies are indicated, and the secondary was used at a dilution of 1:1,000. Images were obtained using a Leica SP2 confocal system. Image acquisition parameters were identical for each collection.

Figure 7. Comparison of resolution achieved using confocal vs. 4Pi microscopy for endogenous human histone H2AX

HeLa cells stained for H2AX were imaged using a confocal (A) or 4Pi microscope (B). Each panel shows the same single xz section from a complete 3D data set. Dotted ellipses mark areas where the enhancement of resolution can be seen best. The insets show the point spread function for each microscope for comparison [re-produced courtesy of the National Academy of Sciences, PNAS 103, 18137 (2006)].

Figure 8. H2AX and γ H2AX show no co-localization at 45 min following exposure of HeLa cells to 3 Gy IR

(A) Sections in all directions through a typical 3D 4Pi data set show no significant overlap of H2AX (green) and γ H2AX (red) signals (dashed lines represent positions of the different sections). Analysis of all data sets by cytofluorograms [example is shown in (B)] reveals a minimal signal overlap (note that color scales are logarithmic). The profiles in the inset (B) represent the cytofluorogram data along the dotted line in B. The fact that they are the same shape agrees with expected coincidental overlap of the resolution-limited signals. The profiles are normalized according to the numbers displayed in the inset (green = 3900; yellow = 200; red = 24). The dotted arrows in the upper left of the cytofluorogram indicate directions for the cross-talk corrected signal [re-produced courtesy of the National Academy of Sciences, PNAS 103, 18137 (2006)].