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Brian Thomas Bennett
University of Massachusetts Medical School

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Immunofluorescence imaging of DNA damage response proteins: Optimizing protocols for super-resolution microscopy

Brian T. Bennett a,b,c, Jörg Bewersdorf d,1, Kendall L. Knight a,*

a Department of Biochemistry and Molecular Pharmacology, Aaron Lazare Medical Research Building, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01655, USA
b Active Motif, 1914 Palomar Oaks Way, Suite 150, Carlsbad, CA 92008, USA
c Biology Department, University of Utah, 257 South 1440 East, Salt Lake City, UT 84112, USA
d Institute for Molecular Biophysics, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA

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 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 proteins can often be ignored in Western blotting procedures, whereas multiple cross-reacting bands appear when using 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 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. Fig. 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 h. At 2 h 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 (Fig. 3A–C), cells treated with specific siRNAs showed nearly complete loss of signal (Fig. 3D–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 (Fig. 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 40×, 63×, Leica 63×: 0.14–0.18 mm). Thickness (0.17 mm) 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 manufacturers 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 30°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, if 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 Fig. 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., 1:1000–1:2000, 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 γH2AX when using an antibody cocktail staining protocol vs. a sequential staining protocol. γH2AX is a phosphorylated version of the histone variant H2AX that in mammalian nuclei appears very rapidly following exposure of cells to DNA damage. γH2AX 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 γH2AX and from methods descriptions it appears that mixtures of primary antibodies, and subsequently secondary antibodies, are used for immunostaining [14,15,31–38]. In Fig. 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 γH2AX (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 h, fixed in 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.

**Fig. 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 stained 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.

In mammals, the Rad51 and H2AX proteins play critical roles in DNA double-strand-break (DSB) repair and DNA damage signaling. The Rad51 protein is essential for homologous recombination, a mechanism used to repair DSBs. H2AX, a histone variant, is a marker for DNA damage and is recruited to sites of DNA damage, including DSBs. The presence of γH2AX in mammalian nuclei indicates DNA damage [14,15,31–38].

The use of antibody mixes or cocktails containing antibodies from different species can enhance or create the appearance of colocalization. It is important to consider these aspects when interpreting immunofluorescence images.

**References**


4. H2AX is a phosphorylated version of the histone variant H2AX that in mammalian nuclei appears very rapidly following exposure of cells to DNA damage.

5. γH2AX has been widely used as a marker for DNA double strand breaks following its discovery by the Bonner group in 1998.

6. In Fig. 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 γH2AX (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 h, fixed in 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 stained 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.
of γH2AX, 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-γH2AX primary antibody. These results suggest that the primary γH2AX antibody out-competes the primary Rad51 antibody for interaction with the secondary antibody. This is most likely explained by the excess epitope availability of γH2AX relative to Rad51 following DNA damage. Therefore, it stands to reason that we could recover the appropriate Rad51 signal by reducing the amount of γH2AX primary antibody used in the staining procedure. The images in Fig. 6C and D show the results of such an experiment. In this case cells were stained using our standard sequential staining procedure and a 1:1000 dilution of anti-Rad51 rabbit primary antibody, but the anti-γH2AX 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 γH2AX epitope available following the response of cells to DNA damage creates a situation where there is such an excess of primary γH2AX 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 γH2AX 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 γH2AX 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 NA 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 (100×/1.35 NA) [22], or alternatively 2,2-ethylenediol and oil immersion lenses (100×/1.46 NA) [51] provides conditions suitable for super-resolution imaging of the nucleus.

In Fig. 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. 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)].](image-url)
(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 γH2AX 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 Fig. 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 1000 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 γH2AX at 100 nm resolution. This is seen in Fig. 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 γH2AX clusters do not colocalize [22].

While 4Pi microscopy provides a 5–7-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 20–30 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 1000-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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