An overview of the visualisation and quantitation of low and high MW DNA adducts using the trapped in agarose DNA immunostaining (TARDIS) assay

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Abstract

The ability to detect and quantify specific DNA adducts benefits genome stability research, drug development and the evaluation of environmental mutagens. The TARDIS assay was developed as a means of detecting and quantifying melphalan and cisplatin DNA adducts at the single cell level and has since been adapted to quantify topoisomerase DNA complexes. The method relies on salt-detergent extraction of agarose-embedded cells. Genomic DNA and any covalently attached molecules remain in place in the agarose, while other cellular constituents are removed. Drug-DNA or topoisomerase-DNA complexes are then detected and quantified by sensitive immunofluorescence using adduct-specific antibodies. Here we give a perspective of the TARDIS assay including a comparison with other methods for quantifying topoisomerase-DNA covalent complexes and provide technical details required to set up and perform the assay.
Original development – quantification of melphalan DNA adducts at the single-cell level

The development of monoclonal antibodies that recognised DNA adducts formed by the anticancer drugs melphalan and cisplatin (1-2) led to the desire to apply these to quantify drug-DNA adducts in individual cells to enable the study of small samples, the assessment of heterogeneity in damage formation and repair within a cell population, and also offered the potential to study spatial distribution of adducts across tissue sections. However, for conventionally fixed cells or tissues there are two challenges to such analyses. Firstly, access of antibody to DNA adducts could be hindered by DNA-associated proteins. Secondly, antibodies often only recognise adducts in denatured DNA. Therefore it was essential to achieve efficient denaturation of the DNA without causing loss of material from the microscope slide. These factors raised concerns about the reliability of any conventional approach to immunostaining because differences between cell types and/or changes to the structure of chromatin in response to DNA damage formation, could affect the efficiency of any immunostaining procedure. Hence, if conventional immunostaining methods were to be used, one would need to be sure that any differences between cells truly reflected differences in adduct levels and not differences in detection efficiency resulting from differences in accessibility to antibody or because it’s DNA was more readily denatured (e.g. there was a nearby single strand break, as discussed below). Another complicating factor is that adduct levels need to be assessed in relation to a reliable measurement of DNA content. Otherwise, one cell could appear as carrying more adducts than another simply because it was in G2 rather than G1 phase of cell cycle or because it was aneuploid rather than diploid.

The technique that was subsequently named the Trapped in Agarose DNA ImmunoStaining (TARDIS) method was initially conceived as a way to overcome these challenges. The idea for the method came to MJT in 1989 whilst watching the preparation of agarose sample plugs for use in pulse field gel electrophoresis in which the intact human DNA molecules are too large to diffuse through agarose and so remained trapped, whilst the porosity of the agarose was sufficient to readily permit diffusion of proteins such as nucleases. If the agarose could be spread on a microscope slide in a thin layer, it might be possible to retain DNA from individual cells whilst achieving efficient removal of essentially all cellular proteins. Such pure DNA would then be amenable to rapid alkaline-denaturation
and subsequent immunofluorescent staining. Also, DNA could be quantified through the use of suitable fluorescent dyes. Finally, the absence of other cellular material could result in lower levels of non-specific staining. Early versions of the COMET assay, available at that time, indicated procedures for spreading and retaining agarose onto microscope slides.

Initial attempts to apply this approach focused on using antibody CP9/19 (2) to detect DNA adducts formed by cisplatin. Initially this worked very well using a standard fluorescence microscope. Immunofluorescence intensity increased in proportion to the cisplatin concentration to which cells had been exposed, from an essentially undetectable level for control cells. It also worked well in the laboratory of Dr. P. Twentyman, where analysis was by confocal microscopy. However, subsequent attempts to apply this method were unsuccessful because of persistent high levels of non-specific binding of primary antibody to DNA from control cells. Despite numerous attempts, this problem could not be overcome. Work then focussed on applying the TARDIS assay to measurement of melphalan-DNA adducts using antibody MP5/73 (1) and in this case the method worked very reliably. It was known from ELISA experiments with antibody MP5/73 that to attain maximum adduct recognition it was necessary to denature the DNA. This property was easily confirmed using the TARDIS method. By having DNA trapped in agarose on a slide it was easy to investigate the effect of different alkali exposures. Such optimisation was important because over-exposure to alkali can cause chemical conversion of melphalan-guanine adducts to the ring-opened form which were known not to be recognised by this antibody. In fact the TARDIS assay was later applied to detect the alkali-induced ring-opened adducts by using a different antibody that does recognise these structures (3). Further studies revealed that optimum detection of melphalan adducts through DNA denaturation could be influenced by the level of strand breaks in the DNA (4). This is explicable, knowing that very large DNA molecules take long times to unwind and fully separate into single strands because unwinding takes place only from the ends. Until it is fully unwound, DNA can rapidly re-nature upon neutralisation (5). An increase in frequency of strand breaks (single or double) increases the number of points from which DNA can unwind thereby accelerating the overall rate of denaturation. Ionising irradiation increased the overall sensitivity of immunological detection of melphalan-DNA adducts and this was attributed to the introduction of strand-breaks. In addition, radiation decreased the extent of inter-cell heterogeneity in immunostaining for melphalan adducts. This indicated that, even in the TARDIS method where DNA is stripped of other macromolecules, differences between cells in
immunostaining can result from differences in DNA conformation rather than differences in actual adduct levels. This illustrates the caution needed in interpreting any immunostaining method for DNA adducts where the binding of the antibody used is known to be influenced by DNA conformation. It seems that the same factors will also influence adduct detection in conventionally fixed specimens, but it is only in the TARDIS method that they can be directly investigated.

**Wider current and potential applications.**

The TARDIS method can, in principle, be applied to any immunologically detectable adduct. Detection of low molecular weight adducts such as those formed by melphalan, are more likely to be affected by DNA conformation. The opposite extreme of adduct size is the covalent binding of proteins to DNA and in such cases it is seems less likely that immunological detection will be significantly influenced by underlying DNA conformation. The most studied examples of protein adducts are the topoisomerase II complexes which are discussed in detail below. However, it could, in principle, be applied to the detection of other proteins that become covalently bound to the genome such as stalled intermediates of other DNA-modifying enzymes and protein cross-linked to DNA by experimental or therapeutic chemicals or by oxidative damage. Here, it is worthwhile pointing out that the TARDIS assay is currently the only available method that permits measurement specifically of protein-DNA adducts in individual cells since, unlike low molecular weight DNA adducts, it is necessary to efficiently remove all of the normally present but non-covalently bound protein whilst retaining all the DNA. Studies referred to above, in which the effect of alkali treatment of melphalan adducts was determined, illustrate the principle that the TARDIS method can permit additional investigations. Since the principle of the method is to allow quite large proteins (antibodies) to access trapped DNA adducts, there is the possibility of carrying out enzymatic as well as chemical alterations to the DNA modifications being investigated. Comparison of immunofluorescent staining with and without such a treatment could provide information about the nature of the target modification such as its susceptibility to specific DNA glycosylases or proteases. For example, enzymatic treatment of COMET slides with glycolases has been reported previously as a means of detecting specific DNA lesions(6).

**Key factors and limitations**

One obvious limitation of the TARDIS method is that it does not permit analysis of cells in the context of tissue architecture in a section. However, analysis of cells microdissected from
frozen sections could be feasible. The staining procedures for melphalan adducts all make use of antibodies of the IgG class. This is likely to be an important property because, in addition to such antibodies tending to show higher affinities, the high molecular weight of antibodies of classes such as IgM could hamper penetration into the agarose. In order to detect low levels of DNA modifications, it is, of course, essential that the antibody shows high affinity for the modification and low non-specific binding. However, unlike conventional immunohistochemistry or immunoblotting procedures, TARDIS preparations contain essentially no cellular material other than the DNA and so the main cause of non-specific binding is absent. More of concern is that there should be very low cross-reactivity with normal DNA. Detection of low molecular weight adducts is more likely to be challenged by this factor than detection of large adducts such as proteins. However, efficiency of detection of proteins bound to DNA, particularly if a monoclonal antibody is used, is likely to be influenced by location of the epitope which could be masked it were close to, or within, the DNA binding site. A range of antibodies have been used for TARDIS to detect topoisomerases. The majority of antibodies that have worked well in TARDIS are rabbit polyclonal antibodies and details are given in the relevant primary publications.

The relative sensitivity of detection for topoisomerase adducts by the TARDIS method is discussed below but, in general, sensitivity is limited by a combination of the following factors: (a) The cross-reactivity and non-specific binding of primary and secondary antibodies. Since the DNA is essentially free of other cellular material, non-specific binding should be very low. (b) Intensity and stability of excitation light. These factors are both optimised by use of a xenon arc lamp or metal halide illumination (see section on microscope specifications below). (c) The back-ground light level in the optics of the microscope. This is determined by the efficiency of the excitation and emission filters and also by features such as the glass and adhesives used to manufacture of the objectives. (d) Accuracy of quantification is determined partly by the stability of the light supply, but also by the uniformity that the light can be distributed to and collected from all regions of the captured image field. Image correction (described below and in ref (7)) can attempt to correct for non-uniformities in these parameters but this far from perfect.

The development of automated laser scanning microscope-based imaging systems offers the prospect of both reduced background light (as a result of confocal optics), higher excitation intensity and more accurate quantification. Also, automated analysis of large numbers of cells
and slides would be a major advantage and would enable high throughput screening for new compounds targeting topoisomerases or testing procedures for potential topoisomerase-mediated genotoxicity.

Unlike the standard COMET assay, the TARDIS method detects specific modifications rather than the presence of general DNA breakage or cross-linkage. It could be of interest to combine TARDIS and COMET assays to investigate the distribution of adducts at different points in the DNA COMET head and tail. Indeed, immunofluorescent staining for topoisomerase IIα has been combined with COMET analysis (8). However, detection sensitivity for adducts in the tail may be limited because detection sensitivity is greatest when DNA concentration is maximised by retaining it in a concentrated spot. In this situation, the ratio of immunostaining to background fluorescence signals is maximal.

**Analysis of topoisomerase II – DNA adducts**

Following its development to quantify low MW DNA adducts the trapped in agarose immunostaining method was adapted to study topoisomerase II adducts. Human DNA topoisomerase II is the target for a number of anti-cancer drugs including etoposide, mitoxantrone, mAMSA, daunorubicin, doxorubicin and idarubicin. These drugs act by stabilising a normally transient DNA-topoisomerase reaction intermediate and are termed topoisomerase poisons (9). Type II DNA topoisomerases generate a transient enzyme-bridged DNA double-strand break through which a second duplex can be passed (strand-passage). At this stage in the reaction cycle the enzyme is covalently coupled to the DNA through a phospho-tyrosine linkage. After strand passage the break is rejoined (10-12). However, the rejoining step is blocked by topoisomerase poisons, leading to accumulation of stabilised topoisomerase II-DNA complexes. The transient reaction intermediates between DNA and the topoisomerase have been referred to as “cleavable complexes” (9) since addition of SDS and proteinase K to these complexes produces a cleaved DNA.

Human cells contain two topoisomerase II isoforms, topoisomerase IIα and topoisomerase IIβ. In vitro cleavage assays showed that both isoforms form drug stabilised cleavable complexes (13-14). To study the isoform-specific effects of drugs in cells we adapted the TARDIS assay to allow isoform-specific detection of topoisomerase II DNA adducts (15), an outline of the methodology is shown in Figure 1. This study showed for the first time that both topoisomerase isoforms could form DNA complexes when human cells
were exposed to etoposide (15). The stabilised complexes increased in a dose-dependent manner and were detectable after a two hour treatment with 1μM etoposide. The rate of disappearance of etoposide-stabilised topoisomerase II complexes after drug removal was analysed and the half life found to be 30 min for topoisomerase IIα and 15 min for topoisomerase IIβ. The TARDIS assay has subsequently been used to assess topoisomerase II adducts levels in human and murine cell lines exposed to a number of compounds listed in Table 1. Both topoisomerase II isoforms formed stabilised complexes with DNA when cells were treated with mAMSA or mitoxantrone. The half life of the mAMSA stabilised complexes following drug removal was 15 minutes for both the alpha and beta isoforms. In contrast the half life of complexes following mitoxantrone removal was 6 hours for beta and 10 hours for alpha. Only topoisomerase IIα complexes could be detected following exposure to anthracyclines such as doxorubicin, daunorubicin and idarubicin. Following removal of idarubicin from the cell culture media the levels of complexes continued to rise for at least 48 hours. Two alkylating anthraquinones, Alchemix and ZP275 preferentially stabilised topoisomerase IIα complexes (16), as did DACA and TAS-103 (17). NK314 a synthetic benzo[c]phenanthridine alkaloid targets topoisomerase IIα preferentially (18). A number of naturally occurring compounds including selenite, curcumin, digitoxin and dietary flavonoids stabilise topoisomerase II complexes and have been analysed using the TARDIS assay assay (18-24) (see Table 1). The TARDIS assay has also been used to detect topoisomerase I complexes in response to camptothecin (25) and other compounds (22-23,26-29). Other groups have used the TARDIS assay (30-32).

Variations

Embedding suspension cells or trypsinised adherent cell culture cells in agarose on a glass slide allows the use of harsh extraction conditions which strips away essentially all non-covalently attached cellular material, leaving genomic DNA and covalent adducts for analysis. This protocol, employed in the TARDIS assay, results in low background signals in untreated cells and corresponding high sensitivity. One variation described by Agostinho et al (33) utilises less harsh extraction conditions to remove most topoisomerase II not covalently attached or trapped in closed clamp form on the genomic DNA from adherent cells grown on glass slides or coverslips. Under these conditions extracted cells and their genomic DNA remain attached without the need for agarose embedding. In our hands this method suffers from higher background levels of topoisomerase fluorescence in untreated cells making it less
suitable than the standard TARDIS assay for quantitative analysis. However, it has been used successfully by Agostinho et al and ourselves to examine the distribution in the nucleus of topoisomerase II – DNA complexes induced by topoisomerase poisons or the topoisomerase inhibitor ICRF193.

**Other methods to study topoisomerase II DNA adducts**

There are a number of other techniques that have been used to detect and quantify topoisomerase - DNA complexes; these include the SDS-K precipitation method (34), alkaline elution (35), ICE bioassay (36) and band depletion (37). The SDS-K precipitation and alkaline elution methods are the most sensitive, however they lack specificity for topoisomerases as any protein-DNA complexes can give a signal and they both also rely on the use of radioactive labelling. The TARDIS assay, ICE-bioassay and band depletion are more specific for topoisomerase, and since they use an antibody detection step they can each be used to detect specific topoisomerase isoforms. The TARDIS and ICE bioassays are comparable in terms of their sensitivity, and both are much more sensitive than band depletion. Band depletion in K562 cells requires >200 μM etoposide to obtain significant depletion of topoisomerase II, whereas the TARDIS assay gives an above background signal with 1 μM for topoisomerase IIα, and a robust signal for both isoforms from 10 μM. ICE bioassay gave good signals at 100 μM (18,36). For topoisomerase I, 25 μM camptothecin gave a 50% reduction in band depletion (26), yet only 1 μM was needed to give a reproducible signal in the TARDIS assay (25-26). 10 μM topotecan gave a strong ICE bioassay signal (36).

The TARDIS assay is the only one of these assays that detects complexes in individual cells and it requires orders of magnitude fewer cells per assay. This makes it applicable for use on samples from patients during therapy where cell numbers are likely to be limited. For example, AML cells treated *ex vivo* with camptothecin showed a dose dependent increase in topoisomerase I complexes (25). Samples from patients after treatment with etoposide have also been analysed (unpublished). The TARDIS assay has also been used to study drug resistant cells lines and to compare the parental sensitive line with the selected drug resistant line. This showed a reduction in topoisomerase II complexes in lines resistant to topoisomerase II drugs (unpublished data).
The TARDIS assay is based on immunofluorescent detection of protein (or DNA-reactive drug) adducts that are covalently coupled to DNA. Such complexes are formed with topoisomerase poisons such as etoposide, mAMSA or mitoxantrone as discussed above. In these cases, the topoisomerase complexes are reversible upon removal of drug. However, topoisomerases (and other chromatin proteins) can also become crosslinked to genomic DNA by compounds that generate reactive oxygen species (ROS). These crosslinked complexes are effectively irreversible in the cell. Thus, the propensity of a compound to generate stabilised topoisomerase-DNA complexes through enzyme poisoning or lead to ROS-mediated crosslinked topoisomerase II-DNA complexes can be distinguished by reversibility studies and the inclusion of antioxidants such as N-acetylcysteine (23).

Comparisons with other methods of assessing genotoxicity indicates that the TARDIS assay has a similar level of sensitivity to the micronucleus assay, comet assay and phosphorylated histone H2AX assay (38). A modified comet assay has been reported where the cells were probed with topoisomerase II antibodies to determine if there were any DNA adducts in the comet tail (8).

There are a range of other uses of the TARDIS assay. It can be used to analyse adduct removal mechanisms in vitro. We have used topoisomerase II adducts on genomic DNA within agarose on slides as a substrate; addition of enzymes to the slides and incubation has been used to determine which proteins are able to remove the topoisomerase II protein. Covalent modifications of topoisomerase could be investigated. Preliminary experiments detected sumoylated proteins (26) and it could be used to detect phosphorylated topoisomerase II or acetylated topoisomerase II if appropriate antibodies were available.

We give details of the preparation of slides for TARDIS analysis, equipment and software requirements and the method of data analysis in sections below.

**TARDIS equipment and procedure**

**Preparing slides**

No specialist equipment is required for the preparation of slides for analysis. A detailed protocol is given in the supplementary material. Briefly, cultured cells are plated into six well plates and exposed to the drug under study. At the end of the exposure period the cells are washed and suspended in molten LMP agarose at 37°. This cell suspension is then spread as a thin layer onto glass slides. The agarose-embedded cells are then extracted with ionic detergent and salt to remove proteins and other molecules not covalently bound to
DNA. After washing to remove salt and detergent, slides are processed for immunofluorescence for the protein or molecule of interest. Counterstaining with Hoechst allows the localisation of extracted nuclei on the slide. The TARDIS protocol is outlined in Figure 1A. Use of a conventional immunofluorescence microscope has proved adequate for many studies, however, to attain useful detection sensitivity and accuracy certain equipment details need to be considered (see more below under microscope and camera specifications).

Typically, the immunofluorescent stage employs an unlabelled primary antibody and fluorescently labelled secondary antibody. We have traditionally used FITC-labelled second antibodies but other fluorochromes such as AlexaFluor488 (Invitrogen) would also be suitable. Monochrome images are recorded for the Hoechst and FITC channels using appropriate narrow-band filter sets for each field of cells. Examples of topoisomerase II TARDIS images collected for etoposide-treated K562 cells are shown in Figure 1B.

**Image analysis**

Image analysis consists of three stages. Firstly, images are background and shade corrected to correct for stray light and background originating in the camera electronics and for unevenness in the intensity of illumination respectively. Secondly, the area corresponding to each nucleus is identified using the Hoechst image to generate a mask around each nucleus. This mask is applied to the FITC image and parameters including number of pixels and average pixel intensity are measured for each masked area (nucleus). In the third stage, these data are exported into a spreadsheet, typically to calculate total FITC and Hoechst intensities measured for each nucleus and this data is represented graphically. An example of the type of output obtained is shown on Figure 1C. We currently use Volocity software (Perkin Elmer) for image management and analysis. A flow diagram for the image analysis is given in Figure 2.

The following points need to be considered carefully for successful quantitative immunofluorescence: (a) **Exposure time.** Exposure times must be sufficiently long to record a robust fluorescent image from the brightest samples, typically in the range of 100mS to 2S for topoisomerase TARDIS, depending on the sample, camera and optics; but the brightest images in a set must not saturate the recording device. This can be checked by taking the sample with the brightest signal or a bright control (for example cells treated with 100μM etoposide for topoisomerase II TARDIS) and recording a consecutive series of images at increasing exposures. Plotting the median fluorescence per nucleus should give a linear response against time. Choose an exposure within the linear part of the response. (b)
**Bleaching.** The above discussion does not take into account fluorescent bleaching of the sample, which may result in lower fluorescent values than expected at longer exposures. Once an optimum exposure is selected in (a) above, record two consecutive images from a single field of cells at that exposure. Both images will give equivalent median fluorescence values unless significant bleaching has occurred. If the second exposure results in significantly reduced fluorescence, consider reducing excitation intensity or use of a more photostable fluorochrome. (c) **Background and shade correction.** It is important to minimise the effect of background signal arising from camera electronics, stray light and background fluorescence. This is achieved by recording FITC and Hoechst images from a blank slide, or preferably a region of the sample slide containing no cells. This “Dark Reference” image is recorded using the same settings including exposure time as the sample slides. This allows subtraction of the background image from the sample images. Any unevenness in the illumination of the sample is minimised using a shade correction. For this an image of an evenly fluorescent sample is recorded. This can be achieved using fluorescent plastic slides or solutions of 3μM fluorescein and 200μM 4-methylumbelliferone in 20μm-deep observation chambers (7). These images are called “Bright Reference” images. The intensity of each pixel in the bright reference image is divided by the mean of all of the pixels in the image. The resulting ratios corresponding to each pixel in the reference image are used to correct the corresponding pixels in each sample image. In practice, software such as Volocity allows the user to assemble combined background and shade corrections for each channel (FITC and Hoechst) using the dark and bright reference images and apply this correction to the sample images in one go. The means of achieving this will differ between software packages. (d) **Internal standards and controls.** It is useful to include a positive control or internal standard in each set of slides to facilitate comparison between individual experiments. We generally include 100μM etoposide-treated cells as a positive control for topoisomerase II TARDIS. An alternative approach would be to use fluorescent beads that are available from suppliers such as Invitrogen.

**Microscope specifications.**

We have used two quite different conventional microscope platforms for quantitative immunofluorescence. The main specific requirements are as follows: (1) good quality planar apochromatic high NA 10X and / or 20X objectives with low levels of autofluorescence associated with the glass and adhesives used to assemble the lenses; (2) a bright stable fluorescent light source ideally a xenon arc lamp or metal halide arrangement such as anX-
cite system (EXFO), suitable narrow band filter sets for FITC (or other fluorochrome) and Hoechst fluorescence and sensitive monochrome cooled CCD camera (see below). Systems can be motorised or fully manual, though considerable time can be saved during image acquisition through partial automation with a motorised system. Specifications for the original system used are described by Frank et al (7) and we also use a similar system based on a Leica DMLB microscope. Recently we have also employed an Olympus IX81 motorised microscope (see Table 2).

**Camera specifications**

Fluorescence arising from DNA adducts is faint compared to typical immunofluorescence of cellular structures in normally fixed cells, so a sensitive monochrome camera system is required. In the system described by Frank et al (4) for the detection of melphalan adducts, images were captured using a cooled slowscan CCD with 16-bit (64,000 greyscale) output and 770x1152 pixels (Astrocam). A similarly specified recent model is the Hamamatsu Orca II BT 1024 (see Table 2). For quantification of topoisomerase DNA complexes, as well as the above-mentioned system we have used both Hamamatsu ORCA-AG and Photometrics Coolsnap HQ2 cameras. Both of these cameras employ cooled CCDs with 6.45μm photosites and 12/14-bit AD conversion. Other required characteristics for TARDIS or indeed any quantitative immunofluorescence analysis include: high quantum efficiency, large photosite full-well capacity and low dark current. These attributes relate to efficiency of converting photons to electrons at the photosite, number of electrons to “fill” the photosite and accumulation of electrons at photosites in the absence of illumination (thermal noise) respectively. Camera models are continually being updated by manufacturers, but some values for the above attributes are given in Table 3 as a guide. Suitable image sensors contain about a million photosites (1 megapixel), and are operated in 2x2 binning mode, whereby 2x2 squares of photosites acts as a single entities.

**Funding**

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**Acknowledgements**
References


Figure Legends

**Figure 1**: Basis of TARDIS analysis (A) Schematic diagram of the steps involved in processing cells for TARDIS analysis: (1) Cells are embedded in agarose on a glass slide; (2) After extraction with SDS and salt most cellular components are removed but genomic DNA and covalent adducts remain; (3) Adducts are detected by immunofluorescent staining using specific primary antibodies and FITC-labelled secondary antibody; (4) Areas occupied by genomic DNA are identified by Hoechst fluorescence; (5) FITC fluorescence is quantified within the areas defined as containing DNA. (B) Representative images recorded for etoposide-treated K562 cells. Immunofluorescence was carried out with anti-topoisomerase IIα and FITC-conjugated second antibody. (C) Scattergram representation of the topoisomerase IIα signal derived from etoposide-treated K562 cells. Each filled circle represents the integrated fluorescence value of a single nucleus. The horizontal lines represent the median values.

**Figure 2**: Flow diagram for TARDIS image capture and analysis.
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Table 1. Examples of TARDIS analyses for topoisomerase IIα or -β poisoning or catalytic inhibition and for topoisomerase I.
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<td>Metal Halide X-cite120Q</td>
</tr>
<tr>
<td>Filters</td>
<td>Narrow band pass “filter cubes”</td>
<td>Narrow band pass “filter cubes”</td>
</tr>
<tr>
<td>Objectives (suitable for TARDIS)</td>
<td>10X HC PL APO NA 0.4 20X HC PL APO NA 0.7</td>
<td>10X VPLA FL NA 0.3.</td>
</tr>
<tr>
<td>Camera</td>
<td>Photometris Coolsnap HQ2</td>
<td>Hamamatsu Orca-AG</td>
</tr>
</tbody>
</table>

Table 2. Microscope specifications used for topoisomerase TARDIS

<table>
<thead>
<tr>
<th></th>
<th>Orca-BTII 1024 Hamamatsu</th>
<th>Orca-AG Hamamatsu</th>
<th>Coolsnap HQ2 Photometrics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosite size</td>
<td>13 x 13 μm</td>
<td>6.45 x 6.45μm</td>
<td>6.45 x 6.45 μm</td>
</tr>
<tr>
<td>No of pixels</td>
<td>1024 x 1024</td>
<td>1344 x 1024</td>
<td>1393 x 1040</td>
</tr>
<tr>
<td>AD conversion</td>
<td>16 bit</td>
<td>12 or 16 bit</td>
<td>14 bit</td>
</tr>
<tr>
<td>Full-well capacity</td>
<td>80K e−</td>
<td>18K e−</td>
<td>16K or 30K e− binned</td>
</tr>
<tr>
<td>Dark current</td>
<td>0.01 e− /pixel/ s</td>
<td>0.03 e− /pixel/ s</td>
<td>0.001 e−/pixel/s (-30°C)</td>
</tr>
</tbody>
</table>

Table 3. Camera characteristics (Data from manufacturer’s datasheets)
Sample slides

Determine optimum (non-saturating) exposure using sample with the strongest signal. (For topoll use 100μM Etoposide). Keep the same exposure for all sample slides.

Blank slide

Record one or more blank-slide images using the same exposure/s as for the sample slides. This is the Dark Reference image.

Hoechst Bright Reference

Acquire FITC and Hoechst images for each sample slide, typically several hundred nuclei per sample over 4-5 fields of cells.

FITC Bright Reference

Record one or more images from the Hoechst and FITC bright reference slides. These are the Bright Reference images.

Microscopy

Image processing software

Use the Dark Reference and Bright Reference images to generate FITC and Hoechst background/shade corrections.

Apply background/shade corrections to FITC and Hoechst images.

Determine areas in image occupied by genomic DNA from the Hoechst image. Record pixel number, area average and total pixel intensities for each area for both the Hoechst and FITC images.

Export data to a spreadsheet.

Plot total (integrated) fluorescence per nucleus.