

Introduction

Symmetrical homodimers of cyanine dyes are known to have exceptional sensitivity for detection of nucleic acids with very high fluorescence enhancement upon binding to DNA. In addition, they exhibit increased quantum yields and stability upon binding due to their high affinity for the nucleic acids. We have recently synthesized a series of homodimeric dyes including 6-chloro-YOYO-1 and 6-chloro-TOTO-1 that are analogs of the commercial dyes YOYO-1 and TOTO-1. They differ from the commercial products in that they have chloro-substituents at the six-position of the benzoxazole or benzothiazole rings respectively. Each dye has also been prepared with different anionic counterions imparting improved water solubility. In addition, their excitation and emission spectra upon DNA binding (EX 450 and 520 nm; EM 510 - 540 nm, quantum, yield 0.5) overlaps well with common fluorescence detection systems. These new analogs have been found to have an approximately two-fold increased sensitivity in agarose gel electrophoresis detection of dsDNA pre-stained with these new dyes over their parent analogs. The new dyes have found application in a number of cell biology assays. Staining of fixed chromatin of human breast cancer cells (MDA-MB-231) exhibited bright green signal, providing an alternate fluorescence detection of chromatin/DNA over DAPI. The new dyes have also found use for post-staining amplification products in Loop Mediated Isothermal Amplification (LAMP) assays. In this assay, we utilized 4 primer sequences which recognize 6 distinct regions of bacteriophage DNA and a strand disrupting polymerase for amplification under isothermic conditions. In addition, upon application at 1 μ M concentration, the new dyes have been found to be suitable for staining flash frozen, ethanol fixed brain tissue sections. Since these new dyes are cell-membrane impermeant, they have been found to be suitable for distinguishing dead cells when used at a 0.5 μ M and therefore act as a vital reagent in Live:Dead format assay. In addition, they are less hazardous than other DNA stains and can thus be utilized in live cell analysis formats. In conclusion, our new 6-chloro-YOYO-1 and 6-chloro-TOTO-1 dyes have increased sensitivity in detection and can be a suitable reagent for a number of important biological assays. This work is funded in part by grant number NSF-IIP0923953 from the National Science Foundation-USA.

Calculation of Binding Affinity

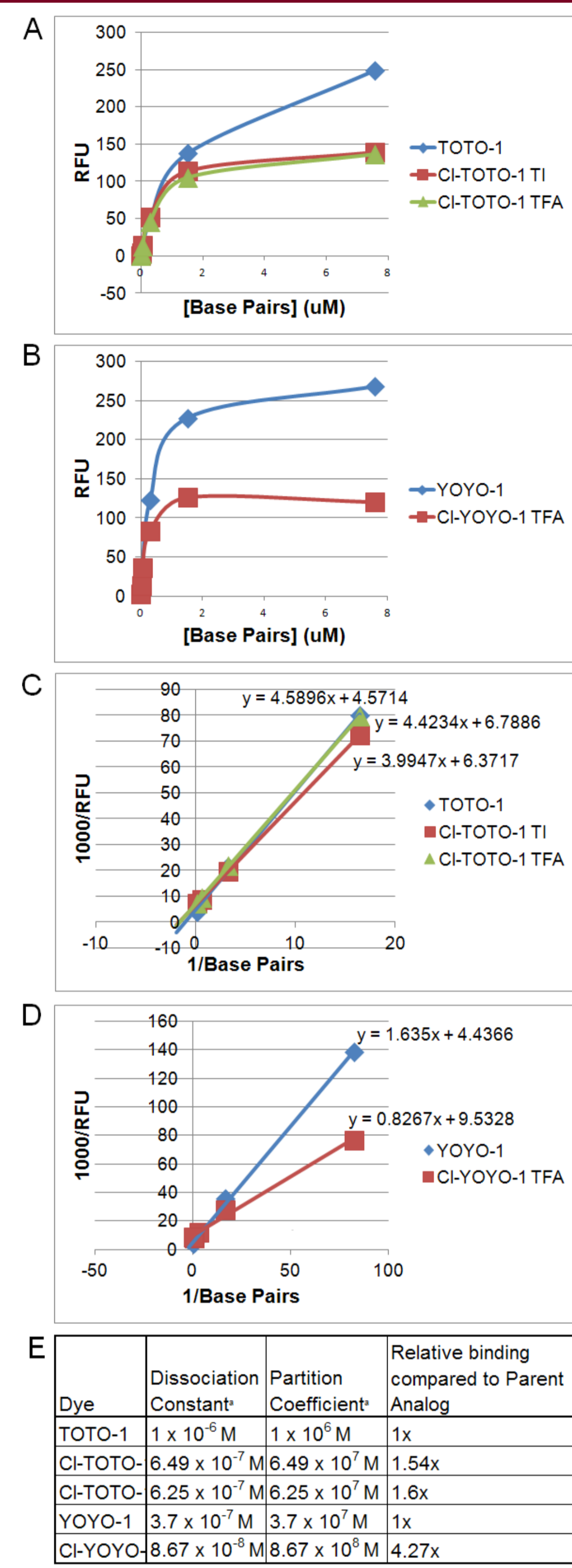
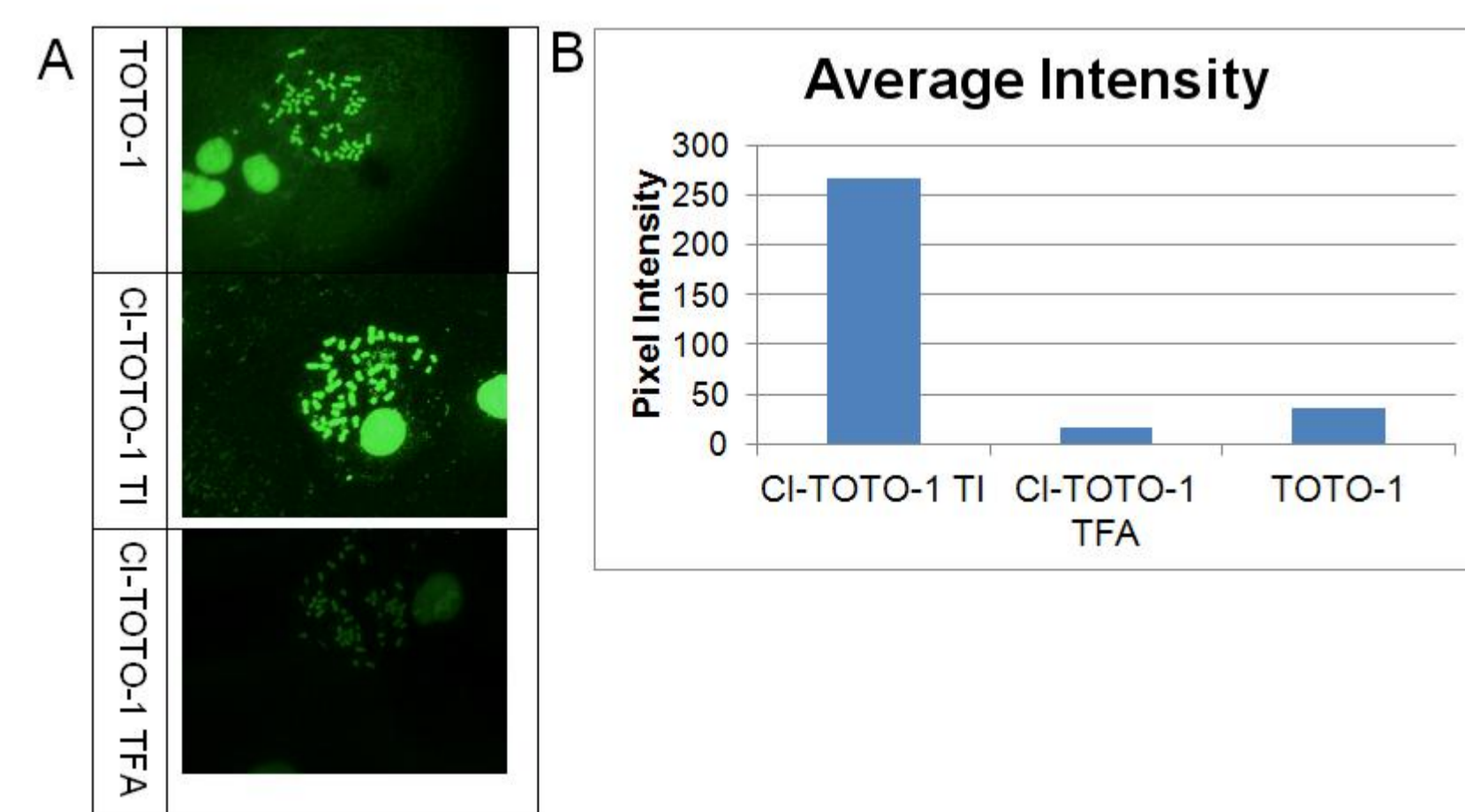


Figure 2. Binding Affinity Binding affinities were calculated by mixing dyes with increasing amounts of DNA until saturation, keeping the dye concentration constant (0.2 μ M) (Graphs A, B). The data was replotted as 1/[Base Pairs] vs. 1000/RFU to achieve linear curves (Graphs C + D). From graphs C and D, the dissociation constant and the partition coefficient (E) are derived using the x-intercept from the equation of the line and then using this in the formula: $K_p = -1/x$ -intercept.

Staining of Fixed Chromosome Spreads



Detection of prestained dsDNA using Agarose Gel Electrophoresis

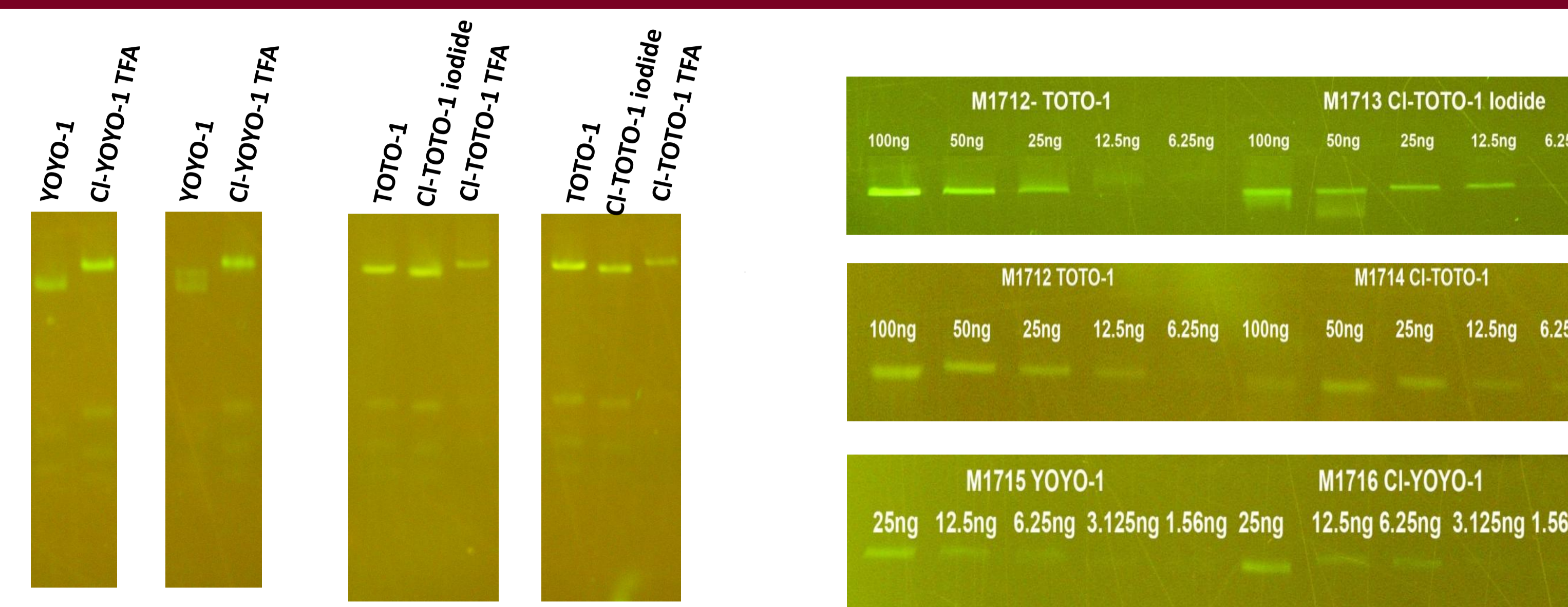


Figure 3. Reduced Incubation Time. The staining properties of CI-TOTO-1 and CI-YOYO-1 dyes were examined by pre-staining BamHI digested fragments of pCMV-Evoglwo-PP1 with the respective dyes and analyzed using 1% agarose gel electrophoresis. 1 μ M dyes were incubated with digested DNA fragments for 5 or 60 minutes before being loaded on the gel. CI-YOYO-1 reached binding equilibrium after 5 minutes unlike YOYO-1 which still displays double bands indicating disequilibrium. Both CI-TOTO-1 dyes also show detection (homogenous bands) after a 5 minute incubation.

Figure 4. Increased Sensitivity. The sensitivity of CI-TOTO-1 and CI-YOYO-1 dyes were tested by pre-staining HindIII digested fragments of pCMV-Evoglwo-PP1 with the respective dyes with analysis using 1% agarose gel electrophoresis. Dyes were used at 1 μ M for pre-staining of the DNA; lower amounts of DNA were titrated by dilution. TOTO and YOYO derivatives exhibited ~2 fold increase in sensitivity in staining DNA.

Detection of LAMP Assay Product by Post-Staining

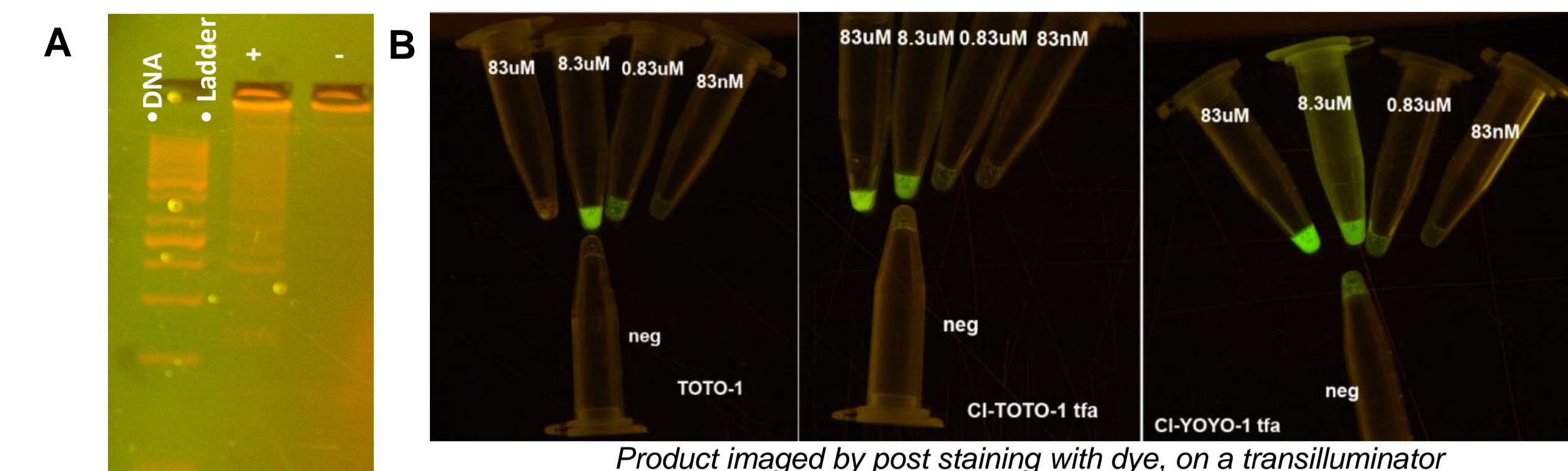


Figure 9: Loop Mediated Isothermal Amplification (LAMP) is a novel amplification method⁹ based on use of 4-6 primer pairs and a strand disrupting polymerase. The reaction is carried out at a constant temperature. We used 2 pairs of primers and a LacZ fragment as template for LAMP assay. The success of the reaction was verified by agarose gel electrophoresis (Panel A), where the ladder product indicates amplification. The amplified product (2 μ L) was added to 10 μ L dye at the concentrations show (Panel B). Both TOTO and CI-TOTO stain LAMP assay products well at high concentrations although TOTO-1 seems to partially quench at the highest concentration. CI-YOYO-1, TFA is more sensitive than TOTO dyes, but it also detects the primers, causing higher background readings.

Staining of Cryostat Tissue Sections

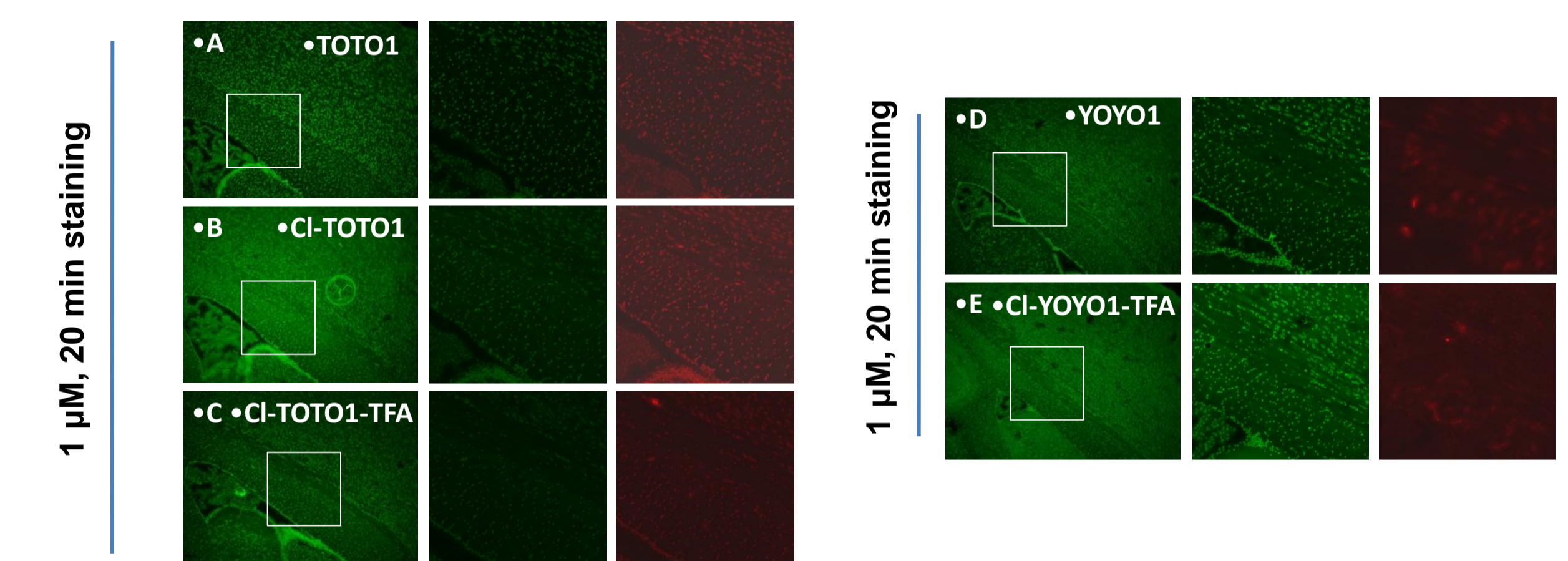


Figure 10: Ethanol fixed mouse brain tissue sections were flash frozen and stained with dyes and their parent analogs as shown. **Figure 10 A,B,C.** TOTO-1 and its derivatives fluoresce in both green and red channels. **Figure 10 D, E** YOYO-1 and its derivative fluoresce well only in green channel.

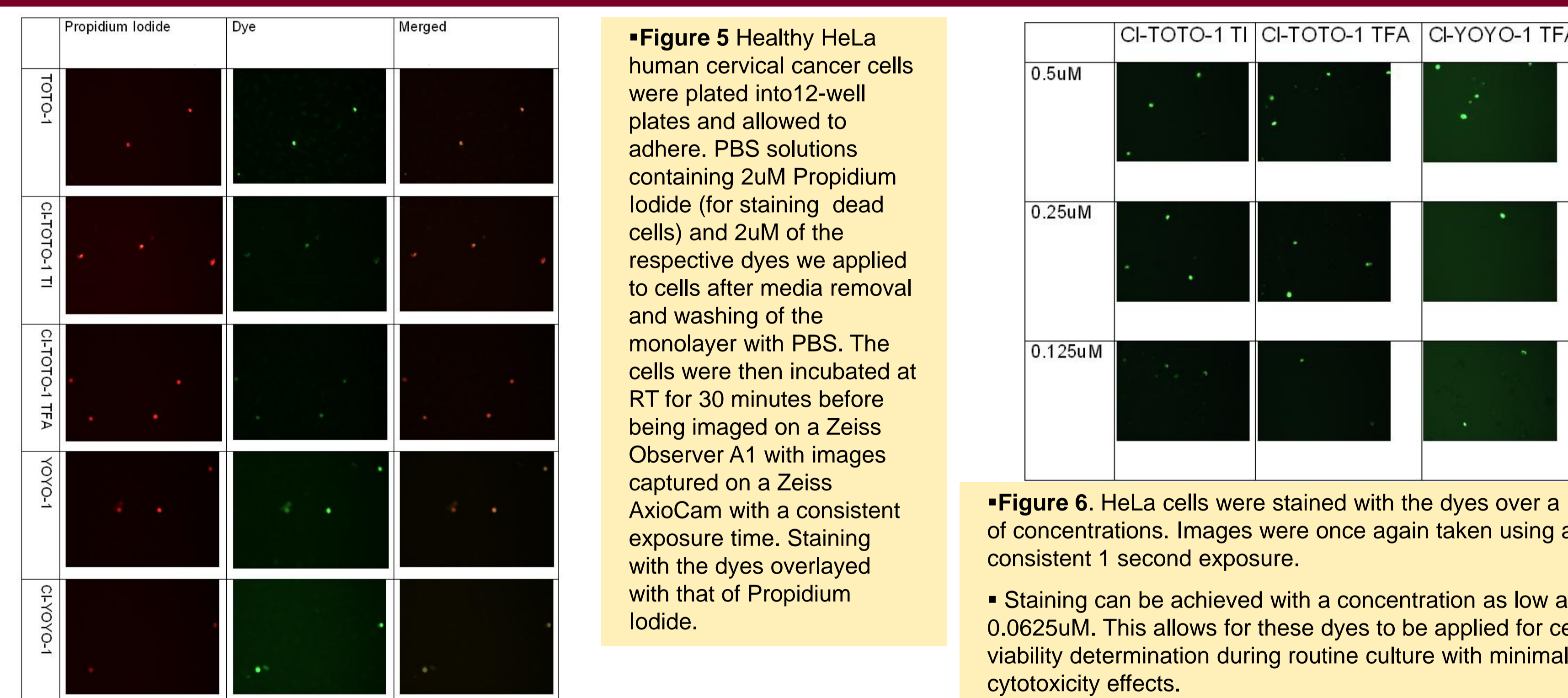
Discussion and Conclusions

We have shown that our new DNA detection reagents exhibit approximately two-fold increased sensitivity over their parent analogs in agarose gel electrophoresis detection of pre-stained dsDNA. As little as 1.5ng of DNA can be visualized using gel analysis. Using fluorometric analysis, as little as 0.09ng/ml of DNA can be detected using these new analogs. The new reagents can be applied to a number of biological assays. Impermeability to cell-membrane makes them suitable for distinguishing dead cells within a culture population, and therefore are utilized as a vital reagent in Live:Dead assay format. We have also shown that staining of dead cells can be achieved with our new dyes at a concentration as low as 0.0625 μ M, making these analogs a less cytotoxic choice compared with traditional reagents. In addition, when used at a 1 μ M concentration, the new dyes have been found to be suitable for staining flash frozen, ethanol fixed brain tissue sections. Furthermore, staining of fixed chromosomes with the TOTO-1 derivatives showed a 5-fold increase in intensity over its parent analog. As these DNA/chromatin dyes emit green (EM 509 or 531 nm), users can have an alternate channel for nuclear detection if red and/or blue emissions have been occupied. These new dyes reagents have also found application in detecting amplification products of Loop Mediated Isothermal Amplification (LAMP) assays. Compared to SyBR Green, the new dyes show significantly less cytotoxicity (~3X) toward cells grown in culture, and are thus safer for use in biological applications. In addition, these new dyes may be suited to live cell assays where the continued culture of the cells is of importance. We conclude that our new 6-chloro-YOYO-1 and 6-chloro-TOTO-1 dyes have increased sensitivity in detection over their parent dyes and have a wide range of applications in biological assays.

References

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Staining of Dead Cells Within a Live Cell Population



Cytotoxicity of the Dyes

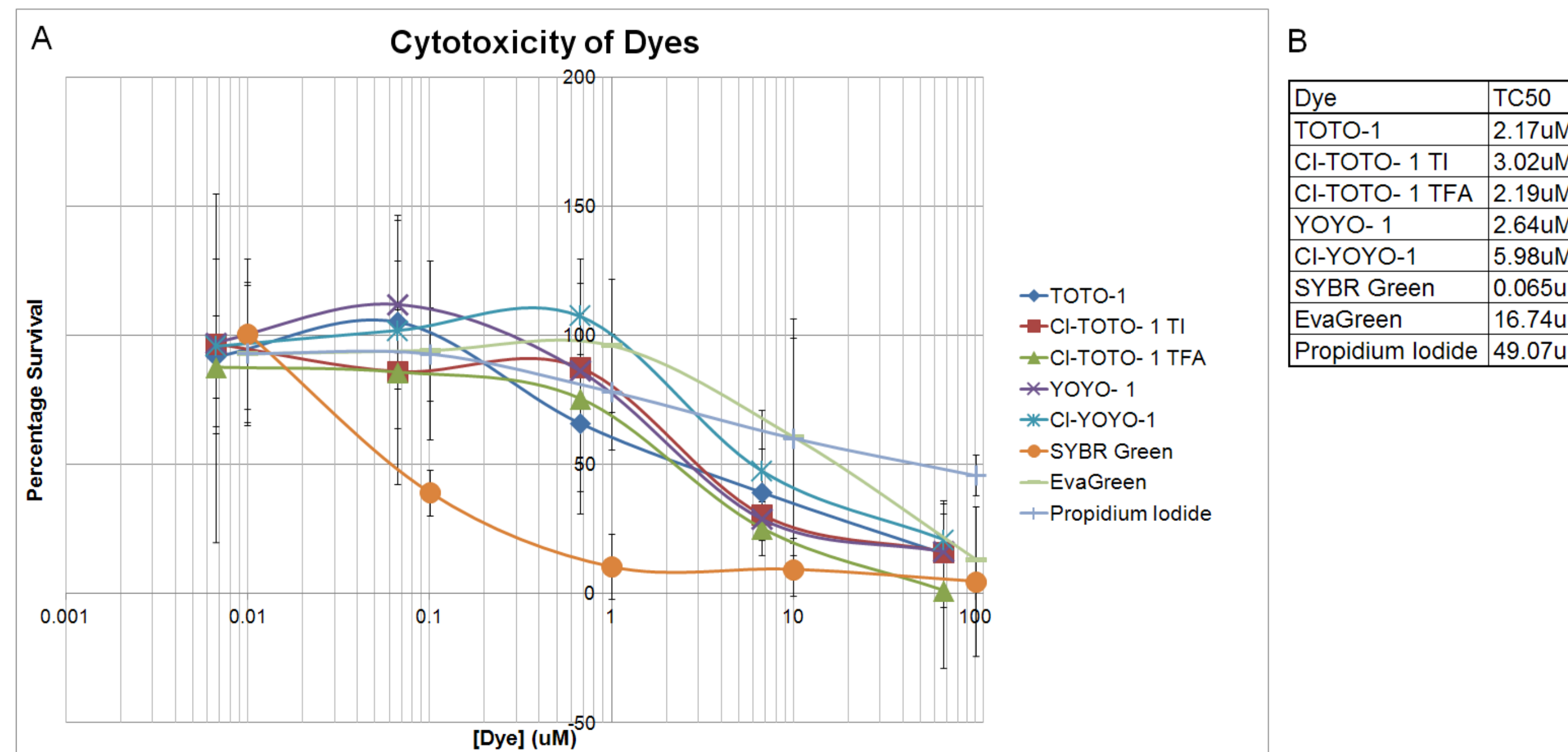


Figure 8. HeLa cells were treated with a varying concentrations of dyes, their parent analogs or other intercalating dyes for 3 days at 37 $^{\circ}$ C, 5% CO₂. The number of viable cells was measured using a standard MTT assay⁶. **Figure 8A.** Percent survival versus dye concentration are graphed on a log scale. **Figure 8B** gives the TC50 values calculated from graph A. All dye analogs exhibit a toxic concentration well above that used in the cell based assays described above.