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Fluorescence detection of nucleic acids and proteins in multi-component crystals

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A highly sensitive and specific assay for the rapid detection of nucleic acids in crystals of macromolecular complexes is reported. The assay is based on the observation of crystals under a fluorescence microscope after incubation with a fluorescent dye that specifically binds nucleic acids. The assay allows clear distinction between crystals of free RNA polymerase II and crystals of the polymerase bound to DNA and RNA or RNA alone, even when the nucleic acids account for only 3% of the crystalline material. Fluorescence detection was also used to monitor the diffusion and accumulation of a fluorescence-labelled polymerase-binding protein in pre-formed polymerase crystals. Additional experiments suggest the general applicability of these fluorescence assays for the characterization of crystal contents.

1. Introduction

A major focus of current structural biology is the crystal structure determination of macromolecular complexes, which gives insights into biomolecular recognition. Of particular interest are multiprotein complexes and complexes of nucleic acid-binding proteins with their target nucleic acids, such as complexes of nucleic acid polymerases with DNA and/or RNA or restriction enzymes with their substrate DNA. X-ray structure determination generally begins with the reconstitution of the complex from purified proteins and nucleic acids. The reconstituted complex is then subjected to crystallization screens. However, the initial crystals obtained from such screens often contain only free protein or free nucleic acids, since many complexes are transient in nature and dissociate under crystallization conditions. Consequently, rapid and reliable methods for the analysis of crystal contents are desirable.

Traditionally, the presence of proteins in crystals is confirmed by electrophoretic analysis of washed crystals. Protein-specific dyes are also available that help to distinguish between salt and protein crystals (*e.g.* IZIT from Hampton Research). More recently, mass spectrometry has frequently been used to confirm a crystal's protein content (Cohen & Chait, 2001) and epifluorescence microscopy has been suggested as a rapid method to detect protein crystals in initial crystallization screens (Judge *et al.*, 2005). Nucleic acid crystals can often be identified by their strong birefringence and the large amounts of nucleic acids in such crystals may even be detected by ethidium bromide staining after electrophoresis. However, nucleic acids in protein complex crystals are more difficult to detect since they are typically present in smaller relative amounts and thus not easily revealed in gels.

In this paper, we describe a simple, rapid and reliable assay for the detection of nucleic acids in macromolecular crystals.

The assay is based on a fluorescent dye that specifically associates with nucleic acids and can be visualized by fluorescence microscopy. A similar experimental set up allowed us to monitor adsorption of a fluorescence-labelled protein into preformed crystals of a large multiprotein complex. Whereas such adsorption of a protein into a protein crystal is limited to exceptional cases, our nucleic acid-staining assay is generally applicable to screen initial crystals for the presence of DNA and/or RNA.

2. Experimental methods

2.1. Fluorescence detection of nucleic acids in crystals

Crystals were prepared as described previously (Deibert *et al.*, 2000; Armache *et al.*, 2003; Kettenberger *et al.*, 2003, 2004). Crystals were harvested in the following solutions: *Ngo*MIV and its DNA complex, 4.0 *M* sodium formate; complete Pol II and its DNA/RNA complex, 200 m*M* ammonium acetate, 150 m*M* magnesium acetate, 50 m*M* HEPES pH 7.0, 5.5% PEG 6000, 5 m*M* dithiothreitol; core Pol II and its RNA complex, 390 m*M* $(NH_4)_2HPO_4$/NaH_2PO_4 pH 6.0, 16% PEG 6000, 50 m*M* dioxane and 5 m*M* dithiothreitol. To ensure complete removal of non-specifically associated nucleic acids, crystals were back-soaked in harvest solution at 293 K overnight. Direct comparison under identical conditions was achieved by placing crystals of free protein and the protein–nucleic acid complex side by side on a glass spot plate in 50 µl harvest solution. This drop was mixed with 50 µl SYBR-Gold dye (Invitrogen) diluted 1:5000 with harvest solution. The time-course experiment was started immediately. Maximum fluorescence was typically reached within 1 h, although prolonged incubation for up to 12 h did not significantly change the results nor did it lead to increased staining of protein-only crystals. Fluorescence microscopic images were collected with a Zeiss Axioskop microscope at 50-fold magnification. A time-course of staining was recorded with a confocal microscope (Leica DM

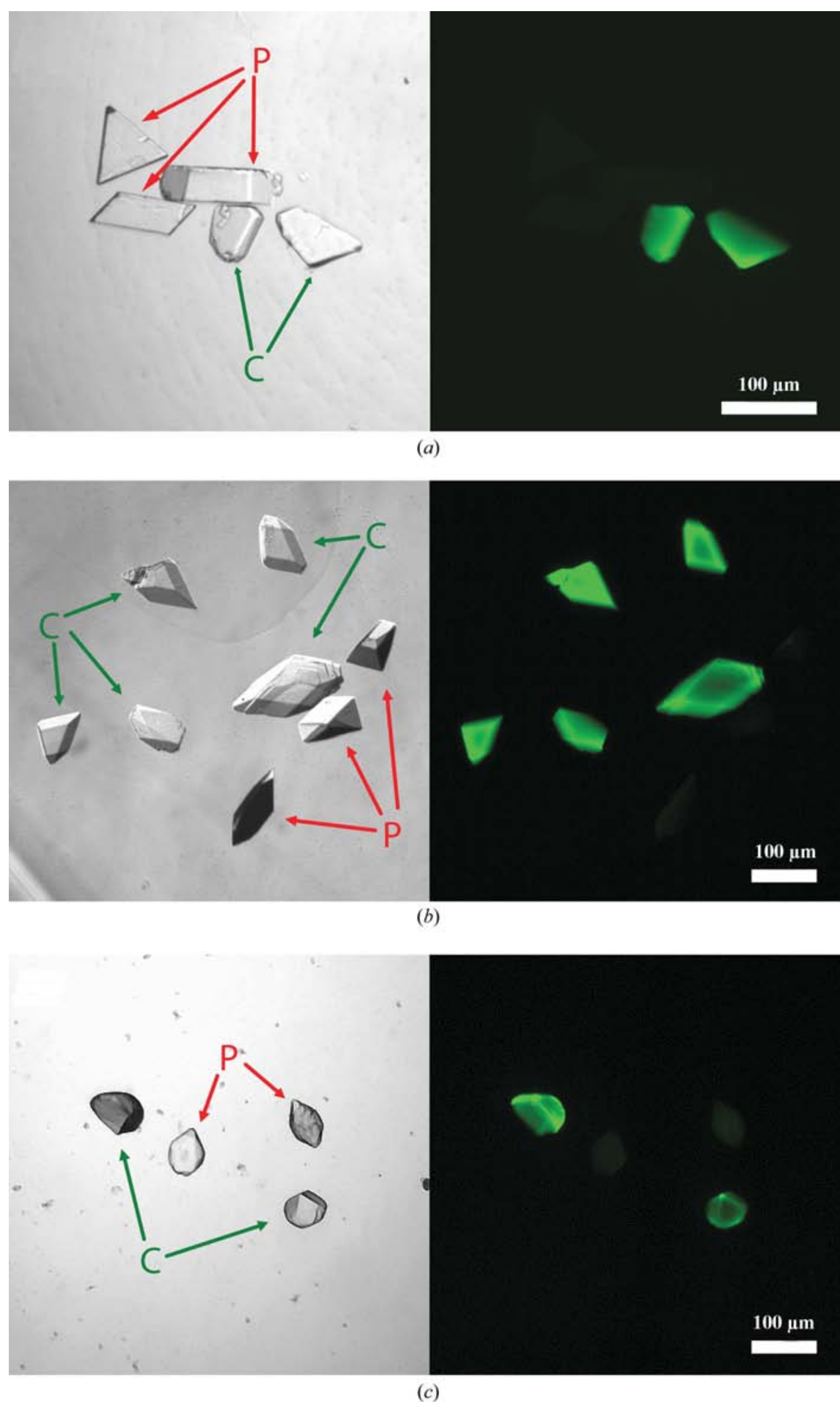

Figure 1
Selective fluorescent staining of nucleic acid-containing crystals. Three comparisons of protein crystals in the free form (P) and in the form of a nucleic acid complex (C) are shown. On the left, transmission light microscopic images reveal all crystals. On the right, fluorescence images show selective staining of nucleic acid complex crystals after incubation with SYBR-Gold. Filters providing an excitation wavelength range of 450–490 nm and an emission wavelength range >515 nm were used. (*a*) Crystals of complete 12-subunit Pol II in the free form and in complex with an 11-mer DNA duplex carrying a 15-nucleotide overhang, which was annealed to an 11-mer RNA (Kettenberger *et al.*, 2004). (*b*) Crystals of core Pol II in the free form and in complex with an 80-mer RNA inhibitor (Kettenberger *et al.*, 2005). (*c*) Crystals of the restriction enzyme *Ngo*MIV in the free form and in complex with an 11 bp DNA duplex (Deibert *et al.*, 2000).

IRE2) at 64-fold magnification. The wavelengths for maximum excitation and emission of SYBR-Gold, 497 and 520 nm, respectively (Invitrogen), are very close to the values for fluorescein (498 and 520 nm, respectively). Therefore, we used filters that are routinely used for observing fluorescein fluorescence. Digital images were recorded with a black-and-white CCD camera (VisiCam, Visitron Systems) or with the built-in CCD camera of the confocal microscope. Integration times were chosen to stay within the dynamic range of the cameras. Image acquisition and pixel integration were carried out with the MetaMorph Imaging System (Universal Imaging Corporation).

2.2. Monitoring TFIIS soaking into RNA polymerase II crystals

To prepare Cy3-labelled TFIIS, 1.0 mg of purified TFIIS in 1 ml carbonate buffer (100 m*M* $NaHCO_3$, 100 m*M* NaCl, 5 m*M* DTT pH 9.0) was incubated with one aliquot of Cy3 antibody-labelling kit (Amersham) at 293 K for 1 h. The reaction was quenched by adding 100 µl of 1 *M* Tris pH 8 followed by incubation for 1 h. Labelled protein was separated from unreacted dye by chromatography over a PD10 desalting column (Amersham) pre-equilibrated with storage buffer (5 m*M* HEPES, 40 m*M* ammonium sulfate, 5 m*M* DTT pH 7.25) and concentrated to 20 mg ml^{-1}. During concentration, no free dye was detectable in the flowthrough by absorption spectroscopy. The resulting molar dye-to-protein ratio was 0.6. Crystals of complete Pol II were cryoprotected by a stepwise exchange of mother solution against a stabilizing solution additionally containing 22% glycerol. Crystals were then incubated for 18–24 h in the same solution additionally containing 2 mg ml^{-1} TFIIS. To record fluorescence micrographs, the microscope described above and a standard Cy3 filter pair were used.

3. Results and discussion

3.1. Fluorescence detection of nucleic acids in crystals

During our efforts to cocrystallize yeast RNA polymerase II (Pol II) with synthetic nucleic acids, we developed an assay for the detection of nucleic acids in our initial crystals. The assay involves incubation of presumed complex crystals with a fluorescent dye that specifically stains nucleic acids and observation of the crystals after staining under a fluorescence microscope with standard FITC filters (compare with §2). Crystals are incubated in mother solution containing trace amounts of SYBR-Gold (Invitrogen), a cyanine dye that binds to any DNA or RNA, irrespective whether it is single- or double-stranded (Tuma *et al.*, 1999). Binding to nucleic acids dramatically enhances the fluorescence yield of SYBR-Gold, whereas proteins have essentially no effect (Tuma *et al.*, 1999).

Crystals of the complete 12-subunit Pol II in complex with DNA and RNA were obtained by incubation of the enzyme with synthetic nucleic acids prior to crystallization and yielded the structure of the Pol II–nucleic acid complex (Kettenberger *et al.*, 2004). We observed that nucleic acid-containing polymerase crystals show bright green fluorescence after 1–2 h incubation with SYBR-Gold, whereas crystals of the free polymerase remained essentially dark under the same conditions (Fig. 1*a*). To test whether the assay also allows for detection of RNA in a protein–RNA complex, crystals of a Pol II–RNA inhibitor complex (Kettenberger *et al.*, 2005) were included in our tests. Once more, the RNA complex crystals were strongly stained, whereas the free polymerase crystals remained essentially unstained (Fig. 1*b*).

To further demonstrate the general applicability of the assay for the detection of nucleic acids in crystals, we applied it to the restriction enzyme *Ngo*MIV cocrystallized with a short DNA duplex, the structure of which is known (Deibert *et al.*, 2000). Crystals of the restriction enzyme–DNA complex were compared with free-enzyme crystals under identical conditions. The *Ngo*MIV–DNA complex crystals exhibited strong fluorescence, whereas crystals of the free enzyme remained essentially non-fluorescent (Fig. 1*c*).

For all nucleic acid complex crystals tested, fluorescence was directly visible under the fluorescence microscope, without the need for digital image processing. Quantified fluorescence intensities for complex crystals were tenfold to 50-fold higher than intensities for free protein crystals of similar size, demonstrating the specificity of the assay. Since crystals of the free proteins were barely stained, even after prolonged incubation with the dye overnight, carrying out the assay in the absence of free protein crystals as a negative control seems a reasonable risk. The sensitivity of the assay is very high, considering the low relative amounts of the nucleic acids in the crystallized complexes. The DNA in the *Ngo*MIV–DNA complex contributes 19% to the mass of the complex, whereas the nucleic acids in the Pol II–DNA–RNA complex account for only about 3% of the complex mass.

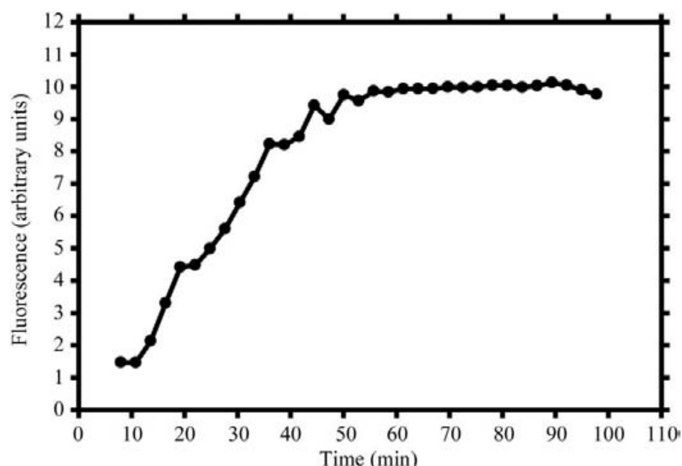

Figure 2
Time-course of crystal fluorescent staining. The fluorescence intensity of crystals prepared under the same conditions as those shown in Fig. 1(*a*) is plotted *versus* time. Confocal fluorescence emission micrographs of a 3.9 µm thick slice were recorded every 168 s with the use of the filter pair described in the legend of Fig. 1. Integration times were chosen to stay within the dynamic range of the camera. The fluorescence intensity was calculated as the average pixel value in a polygon circumscribing the crystal, corrected for the average pixel value of the background. The addition of the dye marked the starting time.

To investigate the kinetics of crystal staining, we recorded a time-course of the increase in observed fluorescence during incubation of the Pol II–nucleic acid complex crystals with SYBR-Gold (Fig. 2). For this experiment only, a confocal microscope was used to visualize a defined layer in the crystal during dye diffusion and accumulation. Under the experimental conditions, saturation of the crystal with the dye was reached after approximately 1 h. Since SYBR-Gold binds rapidly to nucleic acids (Tuma *et al.*, 1999), the slow staining apparently reflects dye diffusion through the solvent channels of the crystals. An implication of this result is that some small molecules can take hours to fully penetrate a crystal and to occupy their binding sites.

3.2. Monitoring protein soaking into crystals

Transient multi-component complexes are often difficult to crystallize, since their reconstitution and purification may

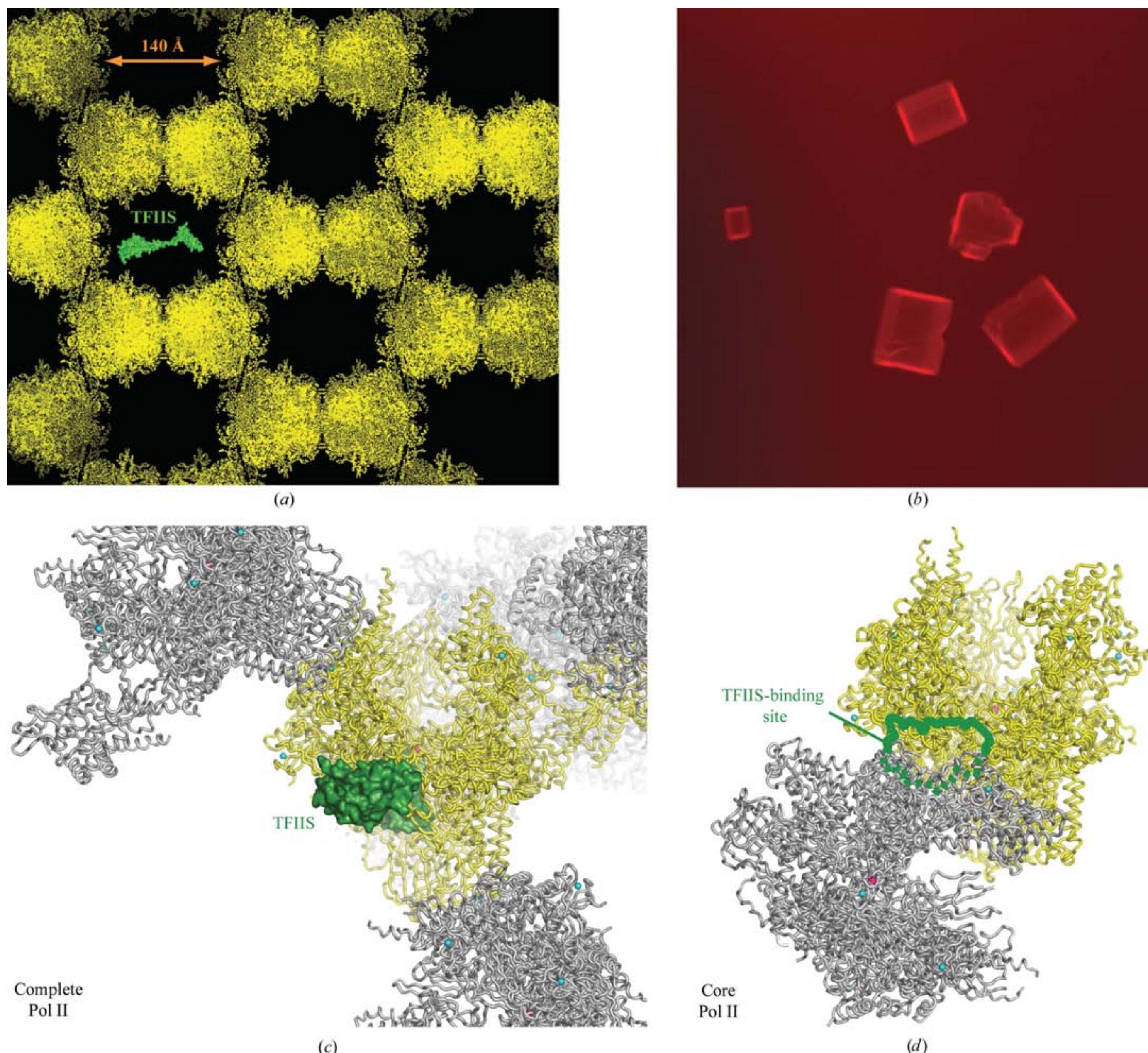

Figure 3
Soaking of Pol II crystals with fluorescence-labelled protein TFIIS. (*a*) Crystal packing of the complete Pol II. Multiple symmetry-related Pol II complexes are shown in yellow, viewed along the crystallographic *c* axis. The molecular surface of a TFIIS molecule is shown inside a solvent channel for size comparison. (*b*) Fluorescence micrograph showing complete Pol II crystals incubated with Cy3-labelled TFIIS for 4 h. Free TFIIS was not removed by washing; thus, fluorescence of crystals above background indicates accumulation of Cy3-TFIIS within the crystals. A filter pair providing an excitation wavelength range of 510–560 nm and an emission wavelength range >590 nm was used. (*c*) In the crystal form of complete 12-subunit Pol II, the TFIIS-binding site on the Pol II surface is accessible. One Pol II complex (yellow) and surrounding symmetry-related Pol II complexes (grey) are shown as tube models. TFIIS is shown as a molecular-surface representation. (*d*) In the crystals of ten-subunit core Pol II (Cramer *et al.*, 2001), the TFIIS-binding site is obstructed by a symmetry-related Pol II complex, explaining why TFIIS binding is incompatible with this crystal form.

coincide with partial complex dissociation. In rare cases, however, this problem may be overcome by diffusing a protein into a preformed crystal of its target complex. To our knowledge, this approach has been successful in three cases. The translation initiation factor IF1 (8.1 kDa) and the trigger factor (17 kDa) were adsorbed into crystals of the small and the large ribosomal subunits, respectively (Carter *et al.*, 2001; Baram *et al.*, 2005) and the transcription elongation factor TFIIS (20 kDa) was adsorbed into crystals of the complete Pol II (Armache *et al.*, 2003; Kettenberger *et al.*, 2004). An advantage of the soaking procedure is that a high concentration of the additional factor in the soaking solution drives stoichiometric complex formation. However, the preformed crystals need to have large solvent channels that allow protein diffusion into the crystal. In addition, the specific binding site of the soaked protein on the target surface must be accessible and not be involved in crystal contacts. We were first prompted to soak additional proteins into preformed crystals of the complete 12-subunit Pol II because this crystal form shows a high solvent content of around 80% and has wide solvent channels of up to 140 Å in diameter (Armache *et al.*, 2003, 2005) (Fig. 3*a*).

To determine whether TFIIS can indeed diffuse into polymerase crystals and whether it can bind to Pol II in the context of our crystals, we covalently labelled TFIIS with the fluorescent dye Cy3 and monitored its accumulation in the crystals by fluorescence (Fig. 3*b*). The fluorescence intensity within the crystals increased around twofold over the background after 4 h. This indicated that TFIIS diffused and accumulated in the crystals. This assay allowed us to test protein soaking in various buffers and with crystals grown under different conditions, resulting in a protocol that led to maximum fluorescence intensity and thus to maximum TFIIS occupancy before crystals soaked with (unlabelled) TFIIS were analysed by X-ray diffraction. We found that the crystals withstand high concentrations of TFIIS, up to 2 mg ml^{-1}, which corresponds to 1 m*M* or ~1000 K_d (Awrey *et al.*, 1998; Sijbrandi *et al.*, 2002; Wu *et al.*, 1996), suggesting that the protocol is applicable to weak and transient protein interactions with dissociation constants in the micromolar range.

We also tried to derivatize crystals of the ten-subunit Pol II core (Fu *et al.*, 1999; Cramer *et al.*, 2000, 2001) with TFIIS under similar conditions. In this case, however, the fluorescence assay showed no accumulation of labelled TFIIS in the crystals (not shown). Compared with the complete Pol II crystals, the latter crystal form has a much lower solvent content of around 50% and a larger area of the polymerase surface is involved in crystal contacts. Comparison of the complete Pol II–TFIIS complex structure with the crystal packing in the Pol II core crystals revealed that part of the TFIIS-binding site on the polymerase is involved in crystal contacts, preventing association with TFIIS in the core Pol II crystal form (Figs. 3*c* and 3*d*). Thus, our results show that the assay can be used to guide protein-soaking experiments prior to crystallographic analysis. An alternative application would be to assemble complexes with one labelled component, crystallize them and assess the fluorescence properties of the resulting crystals in a procedure similar to that suggested by Caylor *et al.* (1999).

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