1 **Single-particle tracking of Nucleotide Excision Repair proteins inside living**

2 **bacteria**

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1  Single-particle tracking (SPT) combined with Photoactivated Localization Microscopy
2  (sptPALM) provides an opportunity to perform complex molecular biology
3  experiments inside living cells. By tracking the motion of DNA repair proteins *in vivo*,
4  information can be extracted not only about their diffusion, but also about the kinetics
5  and spatial distribution of DNA binding[1-3]. From a methodological point of view, a
6  Total Internal Reflection Microscope equipped with a sensitive detector (usually an
7  EM-CCD camera[4]) is commonly used, allowing detection of individual fluorophores.
8  The signal from individual emitters can be analysed and the position of a given
9  fluorophore established with high accuracy (up to a single nm) by Gaussian fitting. To
10  determine the mobility of each fluorophore, the positions of individual molecules are
11  linked into trajectories over multiple frames using a tracking algorithm[5].

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13  Since most proteins in bacteria are present at a copy number, which is too high to
14  resolve individual fluorophores, photoactivable fluorescent proteins (PAFPs i.e.
15  PAmCherry) can be used, allowing the level of active fluorophores to be controlled
16  (e.g. by varying the intensity of a 405 nm photoactivation laser) such that ~1
17  fluorophore is active per cell. This allows for the consecutive observation of all
18  labelled proteins[1-3]. As an alternative to PAFPs, protein tags (i.e. HaloTag), which
19  bind organic fluorophores provided externally can also be used. Once a functional
20  fusion of the protein of interest to a fluorescent label has been constructed, the
21  experiment can begin.

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23  One example of the power of sptPALM was a study of the Nucleotide Excision Repair
24  (NER) pathway in *Escherichia coli*[6]. Fusions of UvrA and UvrB - the proteins that
25  initiate NER, to PAmCherry were introduced into the chromosome and their
26  behaviour was studied in cells, before and after DNA lesions were induced by
27  exposure to UV light. A movie was recorded with 15 ms frame rate and the positions
28  of the fluorophores were localised in each frame and linked into trajectories. For each
29  trajectory, an apparent diffusion coefficient was calculated based on the distance
30  between subsequent localisations[5] (Fig1. A). Molecules bound to DNA showed a
31  minimal change in position, whereas freely diffusing molecules showed large
32  displacements between consecutive frames. The different populations of UvrA and
33  UvrB molecules were quantified by fitting the distribution of apparent diffusion
34  coefficients from tens of thousands of molecules (Fig1. B, C).

1   UvrA was found to bind DNA stably for ~3 s (~40% of molecules) or interact with

2   DNA transiently (low ms range) (Fig1. B). It was proposed that the transient protein-

3   DNA binding is a part of the initial DNA search process, whereas longer binding is a

4   damage verification step. On the other hand, UvrB showed very different behaviour,

5   with the majority of UvrB molecules freely diffusing throughout the cytoplasm (Fig1.

6   C). Cell exposure to UV light caused the recruitment of most UvrA and UvrB

7   molecules to DNA (75% and 60% of molecules, respectively) to repair UV-induced

8   lesions. These sptPALM experiments showed that, in contrast to some historical *in*

9   *vitro* experiments, UvrA and UvrB rarely form a complex in solution; instead, UvrA is

10  a DNA damage sensor, recruiting UvrB to DNA only after damage detection.

11  Furthermore, by using catalytic mutants of UvrA, it was possible to decipher the roles

12  of the two ATP binding sites present in each UvrA molecule, showing that

13  cooperative action in both sites is necessary to recruit UvrB to DNA damage sites[6].

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15  **Cautionary notes:**

16  Previously, one of the key factors preventing the wider adoption of SPT and sptPALM

17  in microbiology has been limited access to the sophisticated equipment and custom-

18  written data analysis tools required for imaging single molecules. However, as these

19  techniques increase in popularity, commercial single-molecule microscopes are

20  becoming more affordable and data analysis tools for SPT are becoming more

21  available[7], opening access to this technique for non-specialist users.

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23  Last but not least, the critical step in all SPT experiments is the construction of a

24  fusion between the protein of interest and the fluorescent tag. Occasionally, this

25  results in an inactive protein. Therefore, the functionality of each fusion protein must

26  be carefully verified. If the fusion is non-functional, the fluorescent tag can be placed

27  at the other end of the protein or the length and nature of the linker can be adjusted

28  to suppress the interference of the tag.

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30  **Conclusion:**

31  SPT is a powerful method, allowing biochemical experiments to be performed in the

32  native environment of living cells. When combined with perturbations such as protein

33  mutations, deletions, or overexpression it can be used to gain deep mechanistic

34  understanding of molecular pathways, and it has been applied not just to the field of

1    DNA repair[2,3,8], but also to study the stringent response[9], transcription[1,10] and

2    translation[11]. SPT is becoming more and more popular, not only in the field of

3    microbiology but also in the eukaryotic field[12]. Furthermore, the availability of user-

4    friendly microscopes and analysis tools[13] will pave the way for STP to become a

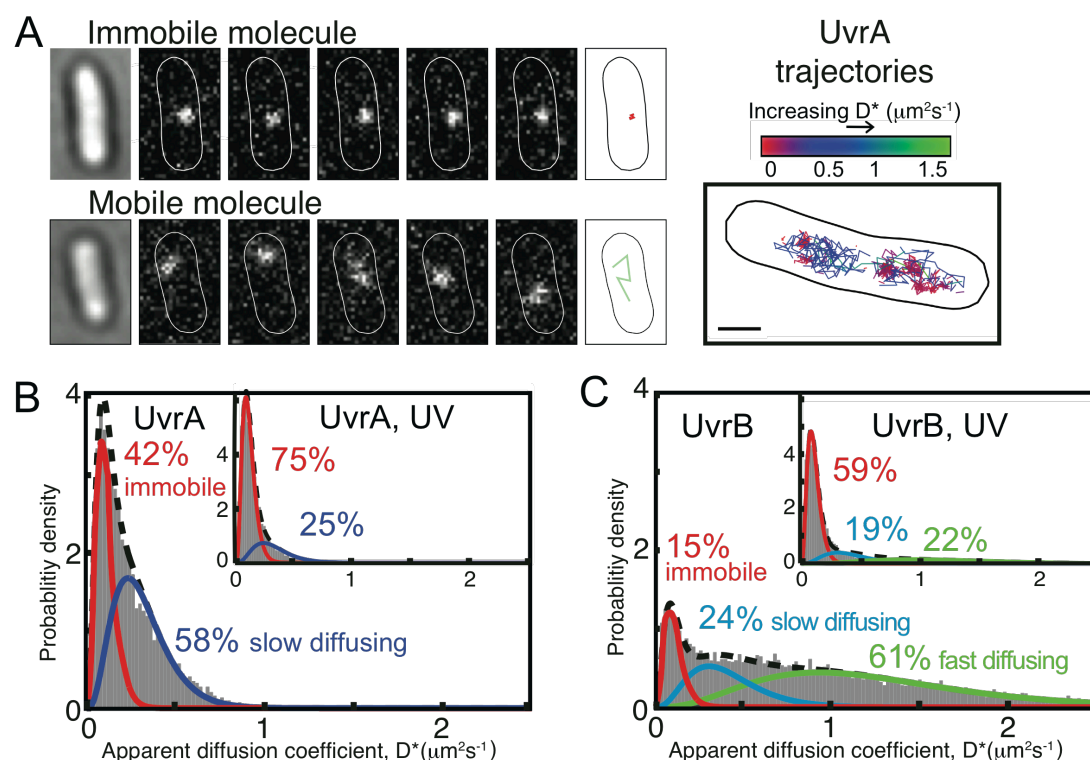5    standard tool in any laboratory.

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9    **Figure 1. In vivo characterization of UvrA and UvrB proteins**

10   *(A) The example image of a single immobile UvrA-PAmCherry molecule localized and tracked at*

11   *15ms exposures within five consecutive frames (top) and the example image of five consecutive*

12   *frames showing fast diffusing UvrB-PAmCherry molecule (bottom). On the right, example cell is shown*

13   *with multiple trajectories recorder for many individual UvrA molecules (B) Distribution of apparent*

14   *diffusion coefficients (D\*) of tracked UvrA molecules, fitted with a two species model: first immobile,*

15   *DNA-bound population (~42%) and second mobile population of slowly diffusing molecules (~58%).*

16   *(Inset) The distribution of D\* values of tracked UvrA molecules after exposure to 50 J m$^{-2}$ ultraviolet*

17   *light (UV). (C) Distribution of D\* values of tracked UvrB molecules, fitted with a three species model*

18   *established that ~15% of UvrB molecules were immobile, ~24% diffusing slowly and ~61% fast*

19   *diffusing. (Inset) The distribution of D\* values of tracked UvrB molecules after exposure to ultraviolet*

20   *light (UV).*

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