Defining off-target cleavage in a pair of Zinc Finger Nucleases

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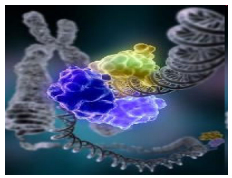
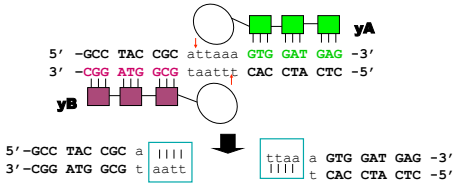


Studies on Zinc Finger Nucleases (ZFN) have shown that they can be toxic in organisms. This is potentially due to ZFN cleavage at multiple off-target sites. In applications of ZFNs in human gene therapy, this off-target cleavage is intolerable. We are attempting to develop a procedure to identify these off-target sites in *Drosophila*. We can then analyze every new ZFN pair for potential off-target cleavage and select and redesign it to work more efficiently.

Our approach is to capture ends created by ZFN cleavage and subject them to deep sequencing using Illumina methodology. ZFN cleavage produces a 5' 4 base overhang at the targeted cleavage site. Because specificity is determined only by the zinc fingers, this overlap will be different at each off-target cleavage site. We have designed adapters with 5' 4 base overhangs which are compatible with Illumina methodology, to capture both ends produced by ZFN cleavage. Analysis of the DNA sequences obtained will be done against the *Drosophila* genome, to identify and characterize off-target cleavage sites for each particular ZFN pair. Previous work in our lab has shown that yellow ZFN pair is toxic in *Drosophila*, thus likely to have multiple off-target cleavage sites. In preliminary experiments, we are able to efficiently capture the targeted ends in an *in vitro* system and in a genomic context. We are working to optimize the procedure, to capture ends created by off-target cleavage. Finally, we will apply the procedure to genomic DNA that has been cleaved *in vivo*.

How ZFNs work



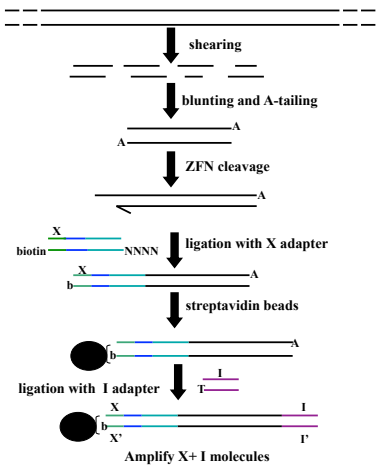
Why study off-target cleavage by ZFNs?

Some ZFNs have been found to be toxic *in vivo* in organisms. This toxicity is largely due to excessive cleavage by ZFNs at off-target sites. Moreover, no extensive examination of *in vivo* cleavage sites has been done and understanding them is needed for ZFNs to be successful as gene-targeting tools.

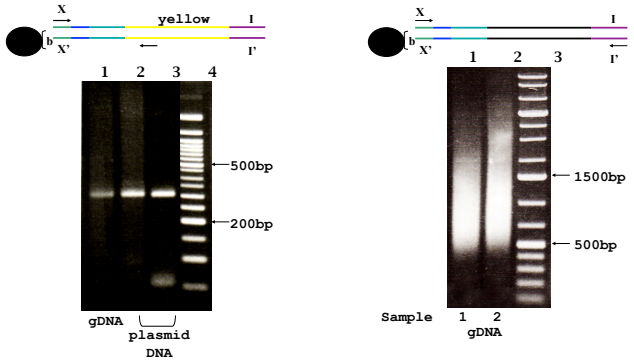
How we propose to examine off-target sites

For getting a comprehensive knowledge of all the sites cut by a pair of ZFNs, we need to be able to specifically select the ZFN-cut ends and enrich them. The standard deep-sequencing methods do not cater to this goal. Hence the need for a customized library-generating protocol which provides specific selection for targets created by a pair of ZFN over the rest of the genomic DNA.

ZFN-produced ends should uniquely have a 4-nt 5' overhang that can be captured with designed adapters.



We have captured yellow ZFN-cut ends *in vitro*



Towards capture of off-targets

We are currently optimizing the steps towards sequencing all the captured off-target sites for yellow ZFNs. Towards that goal, we cloned the X-I' PCR product into pGEM T-vector and sequenced some colonies. We can clearly see cut genomic DNA from *Drosophila* captured between the adapters X and I. Below are some of the sequences. The underlined regions are half-sites of potential off-targets of yellow ZFN and the bases highlighted in red are presumably the 4 bases captured by X adapter.

X- ATAAGAAAATGTTGACTGAACTGTGTTCACAATA.....ACCAAGCTATATTAAGAACTATAGAACACCCCATTCACAACAAGCCGAAATATCGAGACAGA-I

X- GTTGTTTTATTTTTCCAACTGTTCTGAGATTAA.....GCTAGGTGGTTGGGGTGAGAGAAAAGCTTGCTATGATATTTTTCCATAAGTTCTGTGATTG-I

X- CTTGAGAGGGTTAAGAGAGGGTTTAGCTTAGTTGCACAAAGGGTTTAATGGGAATTTTCGATGGGTTATATAAT.....-I

X-CCCGATTCGGAATGTGTCAACGCCACTTCTAAAATGCACAACAAACTGA-I

I'-AATATTGTTCCAACATAAAGGAAACCAAGCCAAACATAATGTAAATGAAGATCTGACCGAACGAAATAATCCAACTATACGACATTATGTCTGTCTTAAAA-X'

I'-CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACTGTGCTCTTCCGATCTGATTTGCATAATATACAA.....GATGTACTTTATGCGCAAA-X'

Doing a BLAST search of the 1st captured sequence shown here, against the known *Drosophila* genome shows that it is on chromosome 2L (11544231-11544406 bp). The region surrounding the ZFN-cut site is shown below:

.....ACACAGTTCAGTCAACATTTTCTTATGTGCAGATGGAAAGTTTTTCTTTAATCTGC.....

.....TGTGTCAAGTCAGTTGTAAAAGAATACACGTCTACCTTTCAAAAAGAAATTAGACG.....

The region around the ZFN-cut site gives us the sequence of the other half-site from the ZFN-cut. In this way, we can analyze the potential off-targets. The above experiment gives us confidence that the method should work on Illumina platform too. We will deep-sequence all ends cleaved by yellow ZFNs *in-vitro* by Illumina methodology. Analysis of the secondary target sites will be done as shown above.

This will give us a comprehensive knowledge of all targets for a pair of ZFNs. Understanding the nature and diversity of the off-targets will help us design more efficient ZFNs.