Tagging Endogenous Genes with Fluorescent Reporters Using CompoZr® Zinc Finger Nuclease Technology

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Abstract

Zinc finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks (DSBs) in DNA at desired locations. DSBs are important for site-specific mutagenesis in that they stimulate the cell's natural DNA-repair processes, namely homologous recombination (HR) and Non-Homologous End Joining (NHEJ). The formation of a DSB increases the rate of HR by a thousand fold between a specific genomic target and a donor plasmid in somatic cells. Sigma-Aldrich CompoZr® ZFN technology is a useful tool to create site specific mutations, and to generate knock-in and knockout model lines for academic and clinical research.

By this approach, we tagged an oncogene, several cytoskeletal genes and a chromatin gene by integrating a fluorescent protein sequence into the desired location (at the sequences encoding either N-terminus or C-terminus of the target protein) in the genome. The integration resulted in endogenous expression of the corresponding fusion proteins (green, red, or blue) that shows their native characteristic pattern. The following five loci were tagged: ERBB2/HER2 (human epidermal growth factor receptor 2, plasma membrane), TUBA1B (α-tubulin 1b, microtubule), ACTB (β-actin, actin stress fibers), LMNB1 (lamin B1, nuclear envelope) and HMGA1 (high mobility group AT-hook 1, nucleus). Single cell clones were isolated in SKOV3 and U2OS, and MCF10 cells with one copy of a given gene tagged. Multiplexing was demonstrated by labeling different genes in the same cell line as well as different alleles of the same gene.

We demonstrate that ZFN mediated gene tagging is an efficient way to generate knock-in cell lines to report endogenous gene expression. It provides the basis for development of various high content screening (HCS) assays for compound screening where target gene regulation and corresponding protein function are preserved.

Introduction

Genome modification of mammalian cells is one of the most challenging and therapeutically important fields that impacts drug-discovery and cell-based assays. At present, gene targeting by homologous recombination is the standard method utilized for precise genome modification. The most efficient approach by far to facilitate HR in the targeted cell is through formation of site specific DNA DSBs. Thus, the ZFN technology has gained popularity in the gene targeting field due to its capability to bind DNA and create a DSB in a sequence-specific manner, dramatically increasing the rate of HR between a specific genomic target and a given donor plasmid.

Materials and Methods

1. U2OS (Cat. No. HTB-96TM) cells and SKOV3 (Cat. No. HTB-77) cells were obtained from ATCC and cultured according to the product manual.
2. Nucleofections were performed with the Amaxa® Nucleofector® device (Cat. No. AAD-1001) and Nucleofector® Kit V (Cat. No. VCA-1003) from Lonza AG according to the product manual.
3. Donor plasmids were designed and constructed in house.
4. Fluorescent reporter genes were obtained from Evrogen (http://evrogen.com/products/TagFPs.shtml).
5. CompoZr® ZFNs were designed and manufactured by Sigma-Aldrich.
6. The cells were imaged with an automated Nikon TE2000-E inverted microscope. BFP: ex 395-410 / em 430-480, GFP: ex 450–490 / em 500–550, RFP: ex 530-560 / em 590-650, 40x/1.4 oil. MetaMorph® was used for image analysis.
7. Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO USA).

ZFN Targeting Mechanism and Donor Design

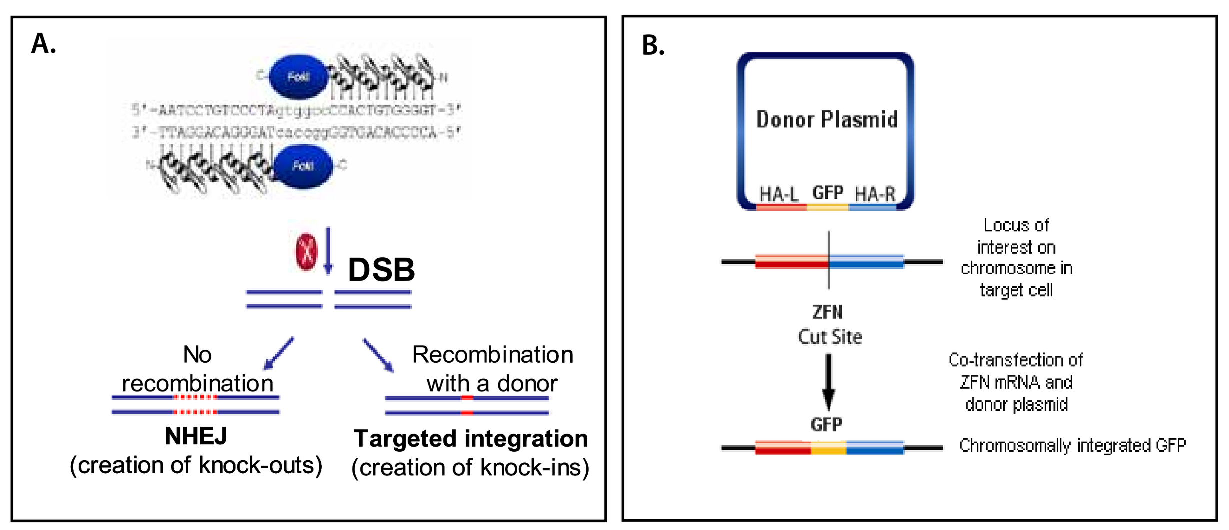


Figure 1: ZFN targeting mechanism and donor design.

A. ZFNs bind to the target site. Then the FokI endonuclease domain dimerizes and makes a double strand break (DSB) between the binding sites. DSBs are repaired by either an error-prone NHEJ pathway or high-fidelity homologous recombination. NHEJ introduces deletions or insertions, which change the spacing between the binding sites so that ZFNs might still bind but dimerization or cleavage cannot occur. In the presence of a donor DNA carrying homology flanking the target site, homologous recombination can use the donor as template to repair a DSB, achieving targeted integration.

B. Generic workflow. The donor plasmid consists of homologous arms (HA-L and HA-R) of the ZFN cut site flanking a fluorescent reporter molecule (GFP).

Results

Successfully Tagged Loci

NM_number (gene name, encoded protein)	Organelle	Human Chromosome Number	Terminus	Distance between ZFN cut site and splice site (bp)	Initial GFP Integration Efficiency
NM_006082 (TUBA1B, α-tubulin 1b)	Microtubule	12	N	7	8.0%
NM_001101 (ACTB, β-actin)	Actin Stress Fibers	7	N	42	9.8%
NM_005573 (LMNB1, lamin B1 - key structural component of the nuclear lamina, an intermediate filament meshwork that lies beneath the inner nuclear membrane)	Nuclear Envelope	5	N	16	1.2%
NM_145899 (HMGA1, High Mobility Group protein HMG-I/HMG-Y isoform A (AT-hook) - a non-histone dsDNA binding protein)	Nucleus (DNA)	6	C	56	0.2%
NM_001005862 (ERBB2/ HER2, human epidermal growth factor receptor 2)	Plasma membrane	17	C	6	0.1%

Table 1: Summary of Tagged Loci in human genome

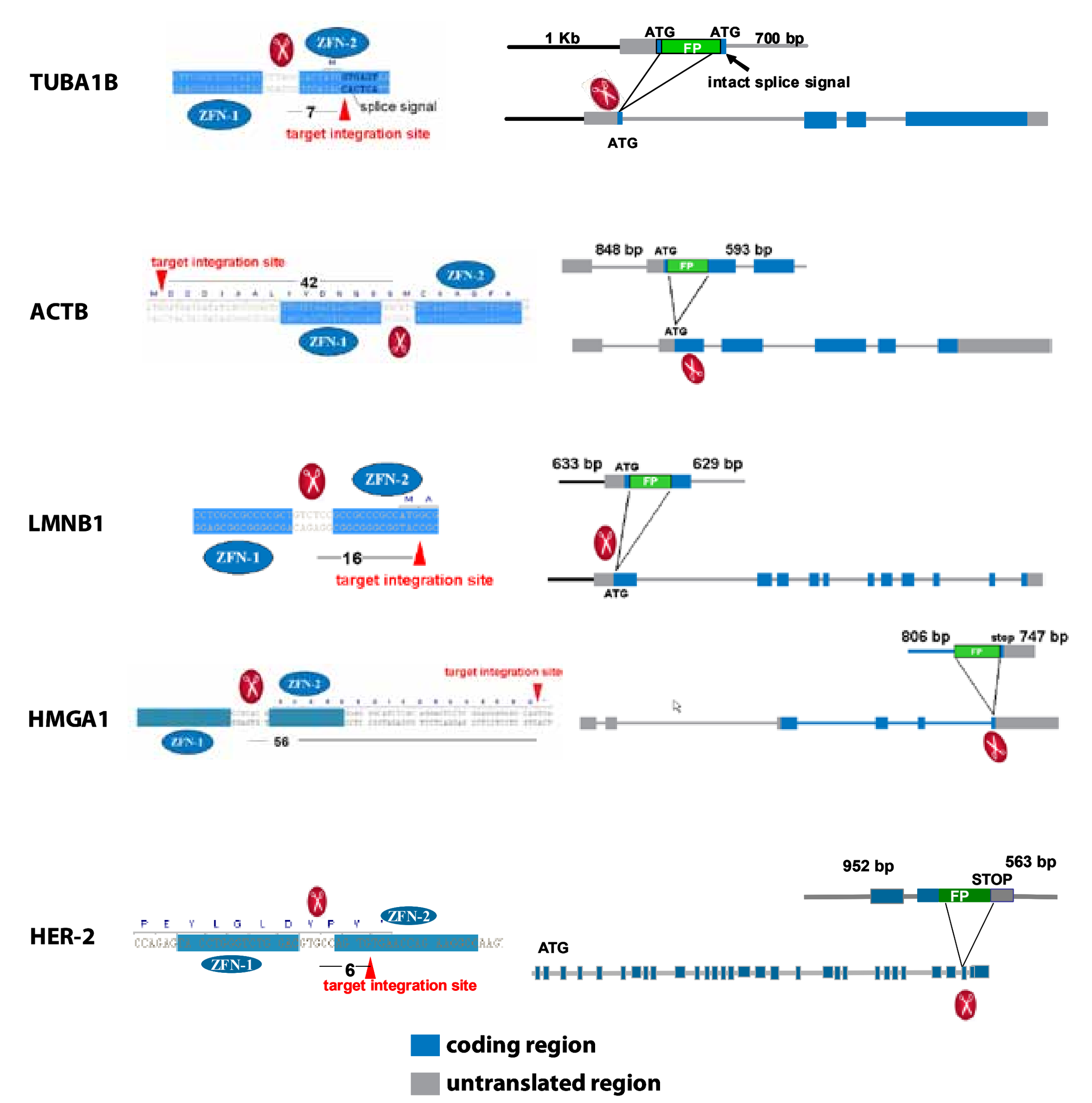


Figure 2. Schematics of the five tagged loci showing ZFN binding sites / ZFN cut site with respect to the targeted integration site. The Donor (top) has the homology arms of indicated length and the FP sequence (green).

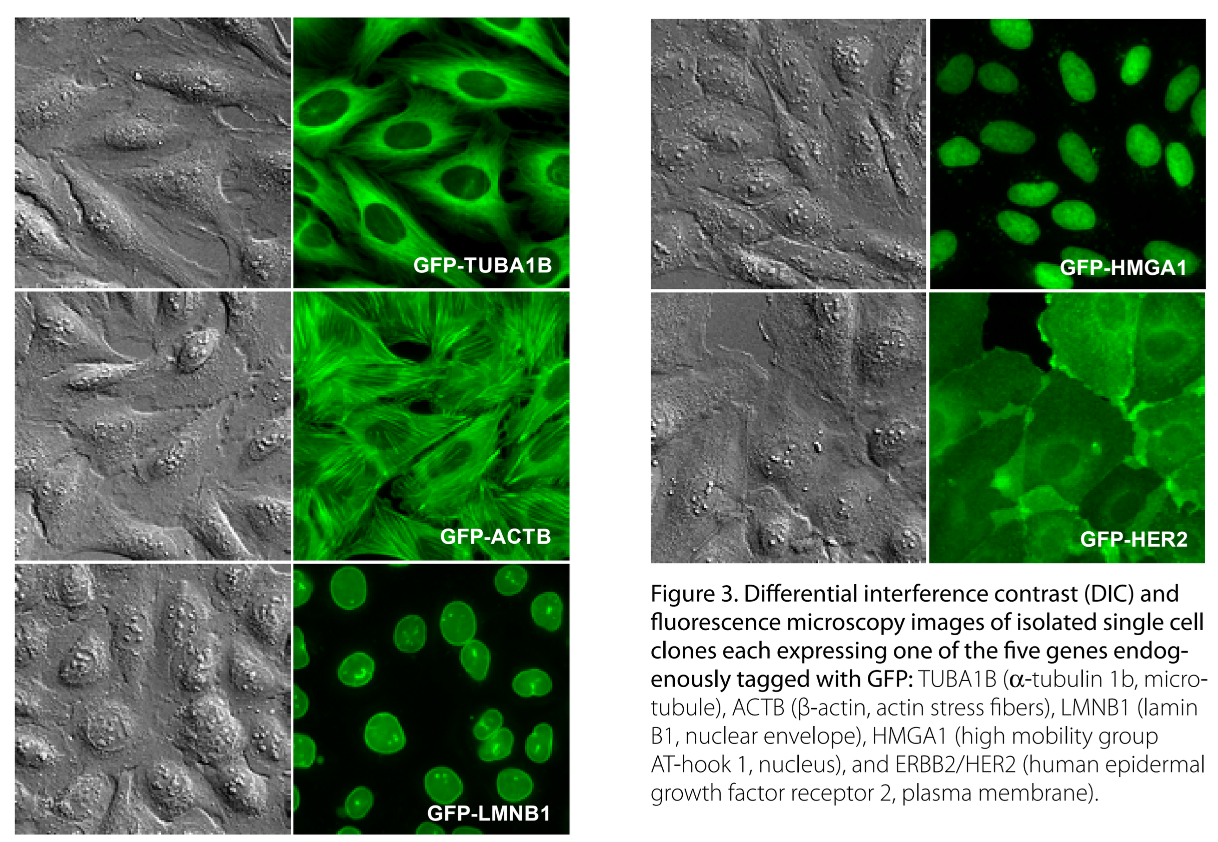


Figure 3. Differential interference contrast (DIC) and fluorescence microscopy images of isolated single cell clones each expressing one of the five genes endogenously tagged with GFP: TUBA1B (α-tubulin 1b, microtubule), ACTB (β-actin, actin stress fibers), LMNB1 (lamin B1, nuclear envelope), HMGA1 (high mobility group AT-hook 1, nucleus), and ERBB2/HER2 (human epidermal growth factor receptor 2, plasma membrane).

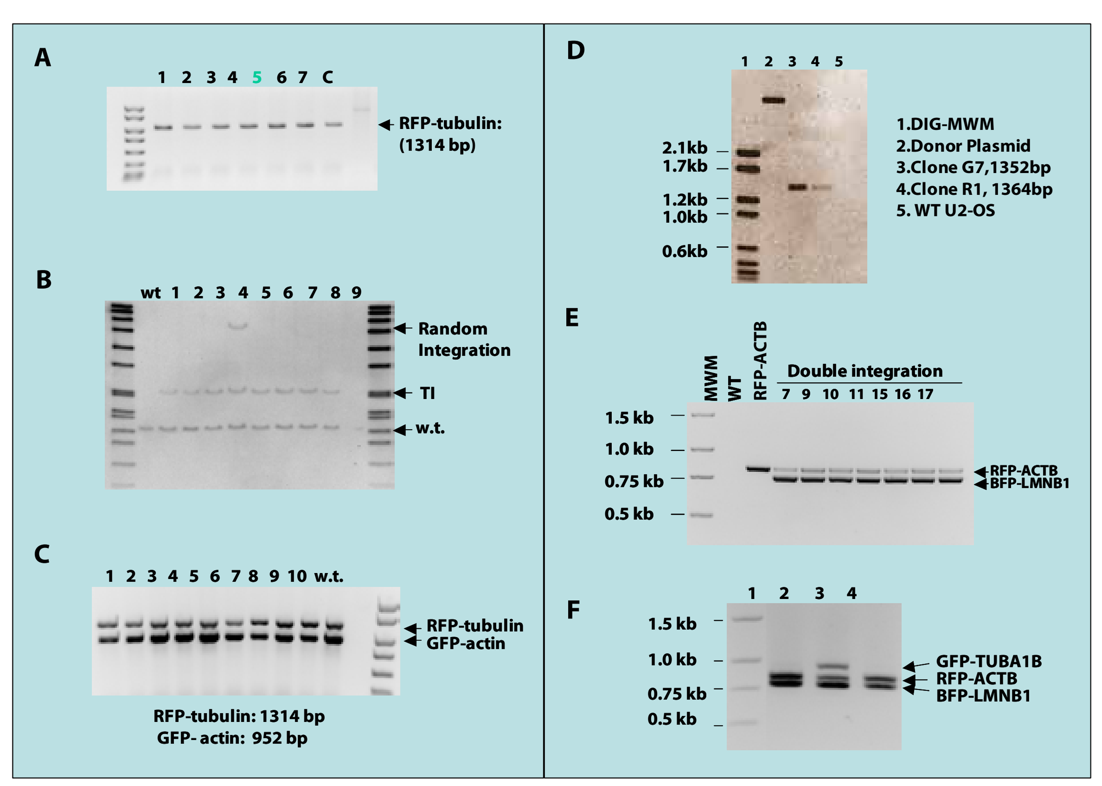


Figure 4. Molecular Analysis. Besides fluorescent imaging, targeted integration was identified by junction PCR and Southern hybridization. In addition, the final clones for all targeted loci were subjected to DNA sequencing to confirm integrated DNA fragments.

Left panel summarizes assays to identify RFP-TUBA1B and its subsequent trait stacking. A. Junction PCR amplification. All 7 clones contained the characteristic integration band at 1314bp. C is the donor-only control. B. Southern hybridizations were performed on PstI digested genomic DNA isolated from wild type U2OS and nine single cell clones positive for red tubulin fluorescence. Using the tubulin probe, a 1946 bp band represents the tubulin genomic DNA with addition of RFP while the 1219 bp band represents wt tubulin genomic DNA. Targeted integration (TI) did not occur at every allele, note the presence of both bands in the lanes 1-9. Out of the 9 TUBA1B-RFP clones, only clone #4 contained an off-target insert. C. RFP-TUBA1B cells were further tagged with GFP-ACTB via ZFN modification (see Figure 8A). The characteristic integration bands for both RFP-TUBA1B and GFP-ACTB were amplified simultaneously by junction PCR in all 10 clones tested, but not detected in wild-type control.

Right panel focuses on some molecular analyses to identify RFP/GFP ACTB and its subsequent trait stacking. D. Southern hybridization, using DIG-labeled GFP and RFP probes, to confirm the single clones for GFP and RFP tagging in ACTB loci. Genomic DNA was digested with NcoI and PstI. G7 is for GFP and R1 is for RFP. E. Junction PCR to confirm double knock-in with RFP-ACTB and BFP-LMNB1 (see Figure 8C). The integration bands for both RFP-ACTB and BFP-LMNB1 were amplified simultaneously in selected clones, whereas only RFP-ACTB can be detected in cells expressing RFP-ACTB and no band can be detected in wild-type control. F. Triple knock-in (see Figure 8E) was confirmed by junction PCR in GFP-TUBA1B enriched cell population expressing RFP-ACTB and BFP-LMNB1. 1- MWM; 2 - Double tagged cells for RFP-ACTB and BFP/LMNB1; 3 – GFP-positive sorted triple tagged population; 4: unsorted triple tagged cells.

Small Molecule Effect

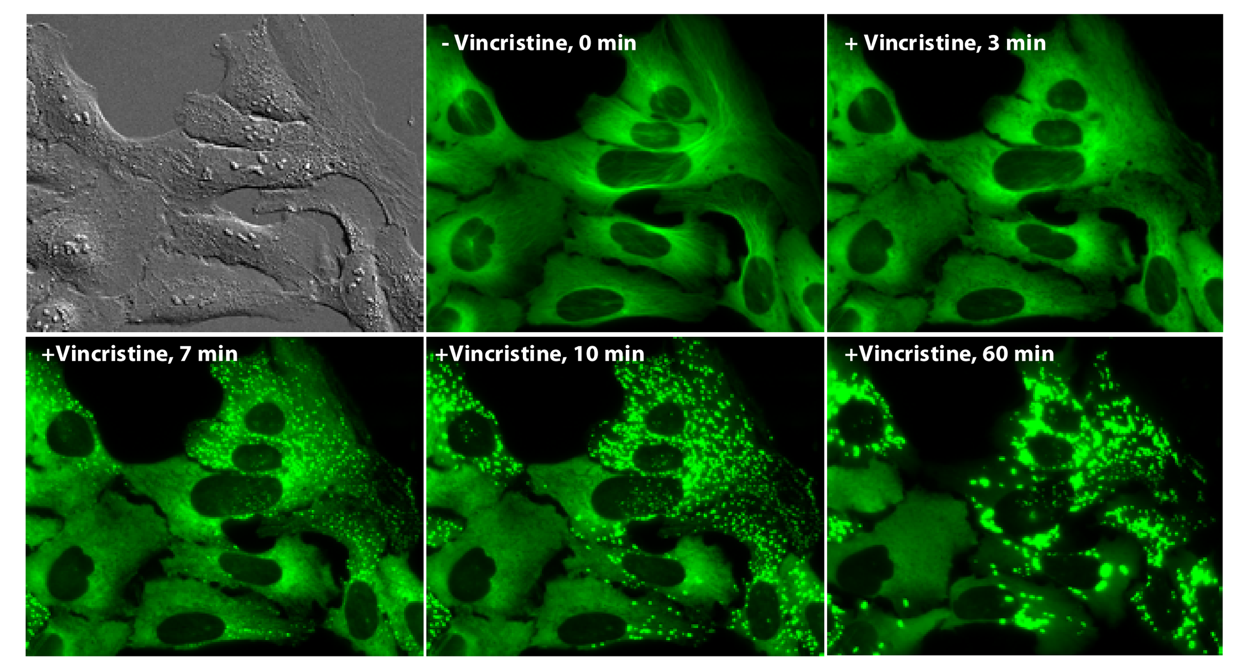


Figure 5. Vincristine effect on microtubules. Vincristine is a mitotic inhibitor used in cancer chemotherapy. Its mode of action is to bind to tubulin dimers thereby inhibiting the assembly of microtubule structures. GFP tagged TUBA1B U2OS cells were exposed to 20 μM Vincristine for sixty minutes. As time progressed, tubulin is repolymerized into a crystalline structure.

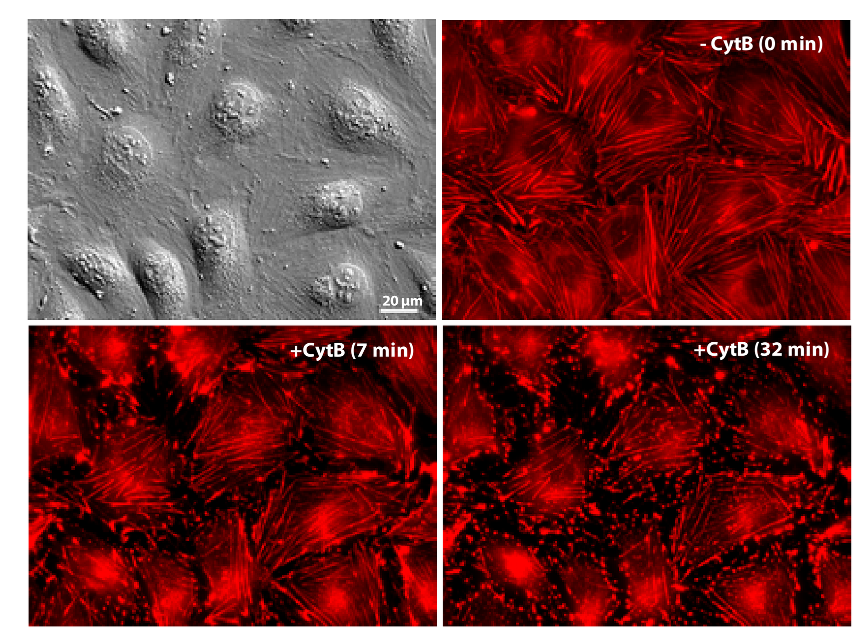


Figure 6. Cytochalasin B effect on actin polymerization. Cytochalasin B is a mycotoxin. It blocks the formation of contractile microfilaments thus inhibiting cytoplasmic division. By blocking monomer addition actin filaments are shortened. RFP tagged ACTB U2OS cells were exposed to 21 μM Cytochalisin B. Over time, shortening of actin filaments can be observed.

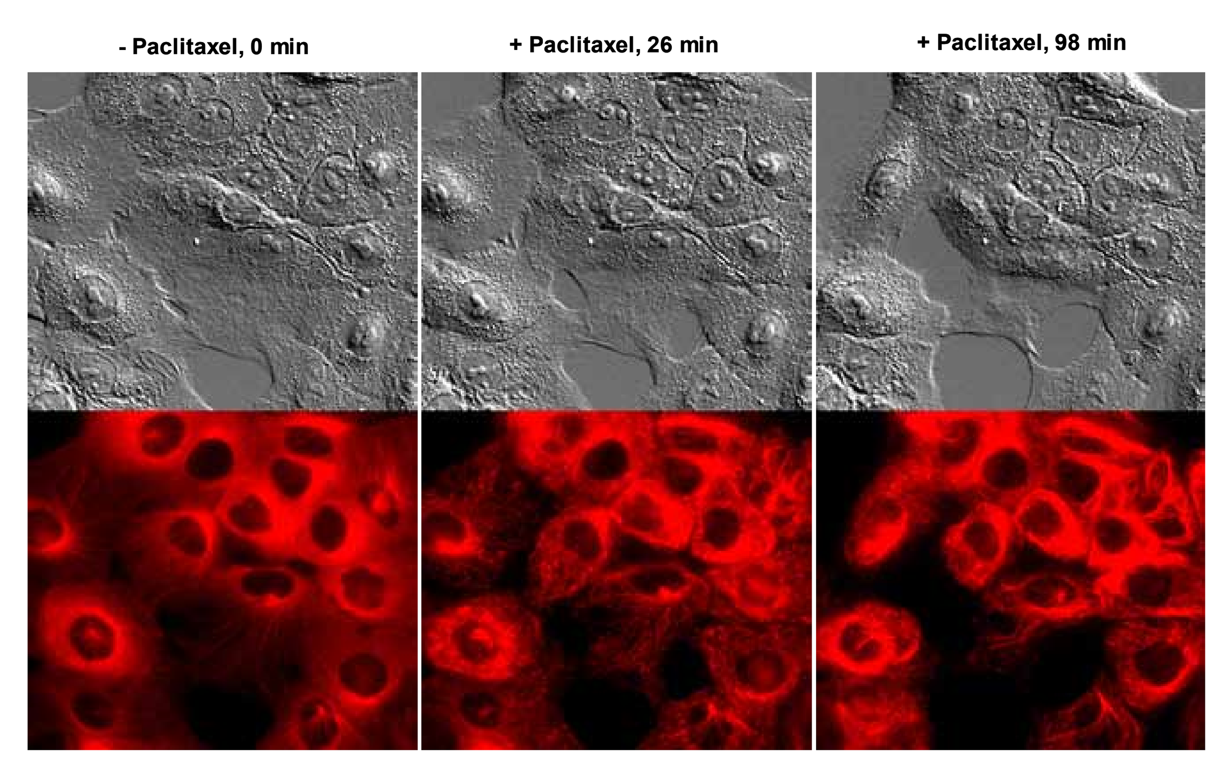
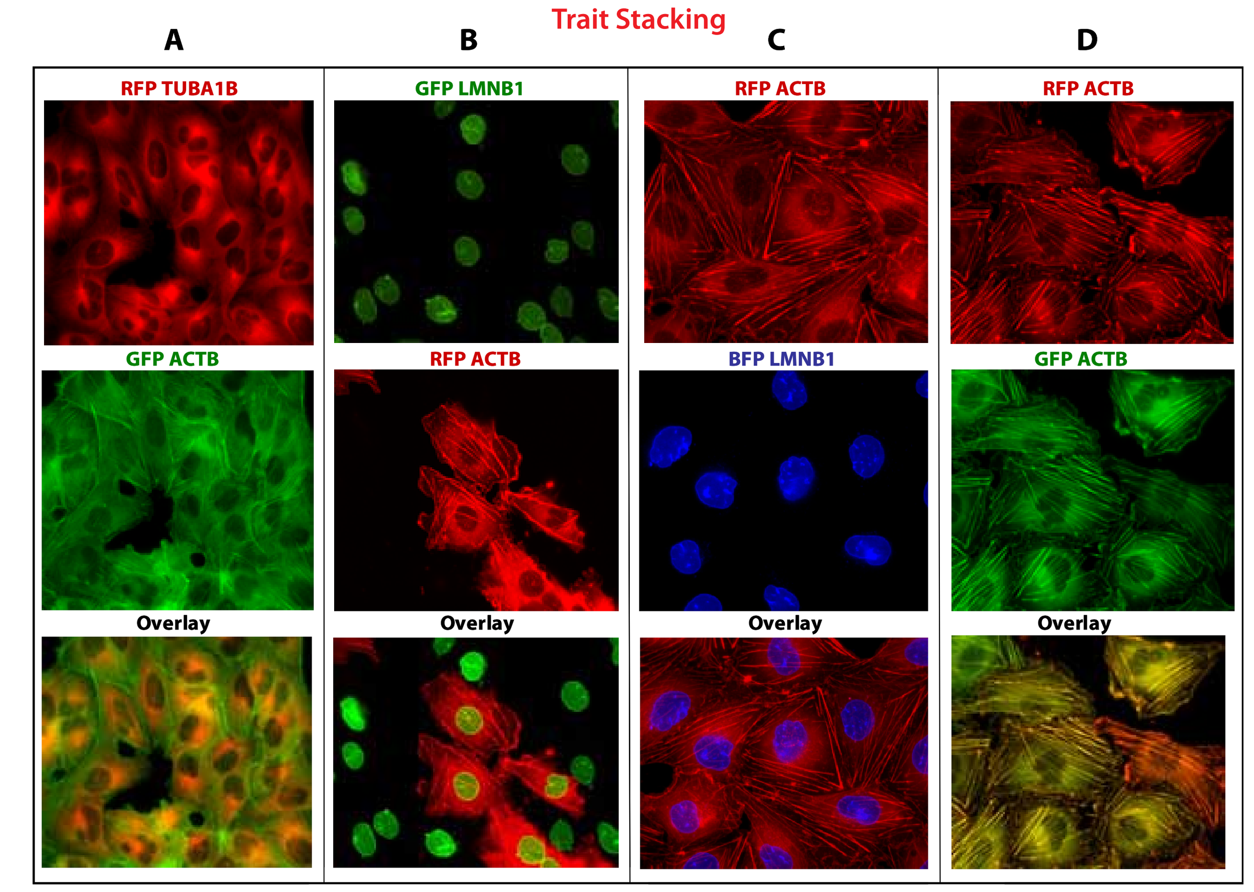


Figure 7. Paclitaxel effect on microtubules. Paclitaxel is a mitotic inhibitor used in cancer chemotherapy. Paclitaxel is thought to stabilize microtubules and as a result, interfere with the normal breakdown of microtubules during cell division. RFP tagged TUBA1B MCF10A cells were imaged by differential interference contrast (DIC) and fluorescence microscopy while applying 20 μM paclitaxel. As time progressed, typical tubulin bundles are formed.

Trait Stacking



E. Triple Knock-In:
tagBFP-LMNB1
tagGFP-TUBA1B
tagRFP-ACTB

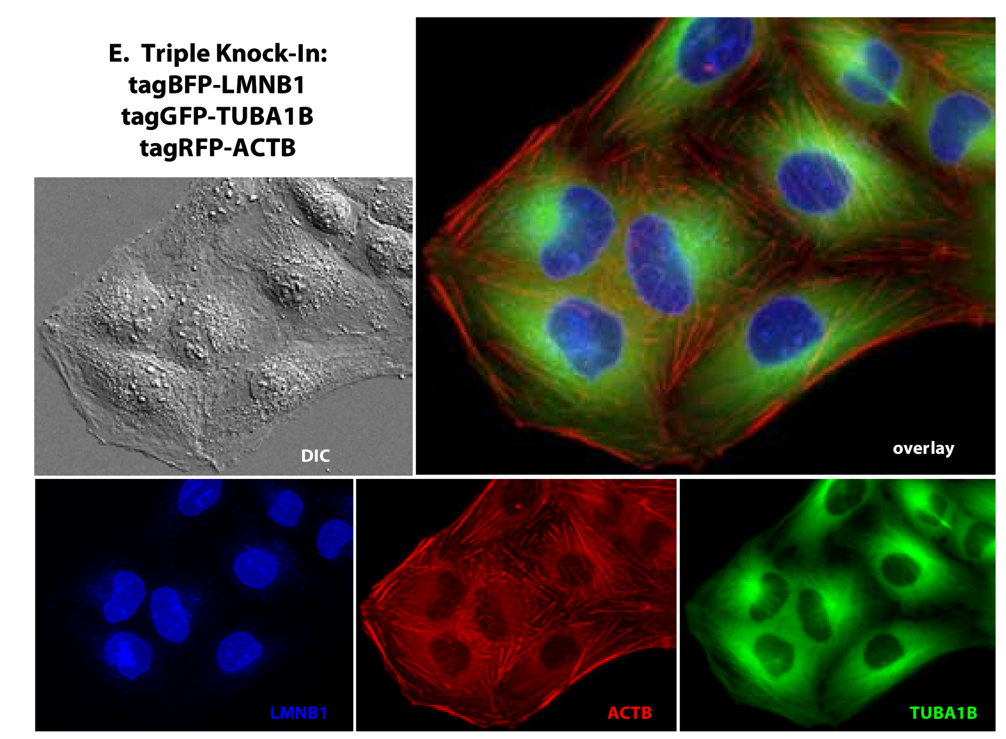


Figure 8: Trait Stacking A. A RFP TUBA1B expressing cell line was modified with ZFNs to also express GFP ACTB. B. A GFP LMNB1 expressing cell line was modified with ZFNs to also express RFP ACTB. C. A RFP ACTB expressing cell line was modified with ZFNs to also express BFP LMBN1. D. The ACTB gene has 3 alleles in the U-2 OS cell line. ZFNs were used to express GFP ACTB in a cell line already expressing RFP ACTB resulting in the modification of two alleles of the same gene. E. U-2 OS cells expressing both RFP ACTB and BFP LMBN1 described in C were further modified with ZFNs to express GFP TUBA1B.

Conclusions

Until now, fluorescence detection of proteins relied on either exogenous promoters or immuno-techniques requiring cell fixation. With the ZFN technology, it is now possible to create stable integration of a reporter gene into the genome. Unlike fusion proteins generated with an external promoter, the fusion proteins created using the ZFNs are expressed at their physiological level and apparently retain the characteristic expression profile of the endogenous proteins in the cell. The fusion protein can be observed throughout the cell's life cycle.

This work shows successful tagging of five individual loci : TUBA1B (α-tubulin 1b, microtubule), ACTB (β-actin, actin stress fibers), LMNB1 (lamin B1, nuclear envelope), HMGA1 (high mobility group AT-hook 1, nucleus), and ERBB2/HER2 (human epidermal growth factor receptor 2, plasma membrane). Labeling of more than two different genes in the same cell line as well as different alleles of the same gene are also demonstrated. Future work includes the study of cellular processes, compound screening, and cell-based assay development.

Acknowledgements

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Related Products

Product#	Species	Description
CLL1031	human	U2OS GFP-TUBA1B
CLL1032	human	U2OS GFP-ACTB
CLL1033	human	U2OS GFP-LMNB1
CLL1034	human	U2OS RFP-TUBA1B
CLL1035	human	U2OS RFP-ACTB
CLL1036	human	U2OS GFP-HMGA1
CLL1037	human	U2OS GFP-ACTB RFP-TUBA1B
CLL1038	human	U2OS BFP-LMNB1 RFP-ACTB
CLL1039	human	MCF10A RFP-TUBA1B