Targeted Integration of Fluorescent Reporter Genes Utilizing Zinc Finger Nucleases

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Introduction

Engineering of the mammalian genome is an area of basic research that also impacts drug-discovery and cell-based assays. This can be accomplished using zinc finger nucleases (ZFNs) – synthetic, chimeric proteins engineered to bind DNA at a sequence specific location and cleave across both strands. The cell's natural machinery repairs the break in one of two ways: non-homologous end joining (NHEJ) or homologous recombination. Employment of the homologous recombination pathway was used to insert a (donor) transgene into a specific, desired location in mammalian cells.

ZFNs were customized/designed to cut near the desired site of integration for four organelle specific genes. These ZFNs were nucleofected along with a donor construct containing a fluorescent reporter gene flanked by sequences homologous to the target integration site into U-2 OS cells. Integration resulted in endogenous expression of fluorescent fusion proteins that labeled a particular organelle.

Four gene loci were tagged: *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). Fluorescent proteins were used as reporters. Integration of the donor fluorescent reporters was genomically stable. Preservation of protein function and gene regulation was observed. Additionally, due to the specificity of the targeted integration process, multiple reporter genes were successfully integrated into the U-2 OS genome.s.

Methods

U-2 OS (Cat. No. HTB-96TM) cells were obtained from ATCC and cultured according to the product manual. 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. Donor plasmids were designed and constructed in house. Fluorescent reporter genes were obtained from Evrogen (http://evrogen.com/products/TagFPs.shtml). CompoZr® ZFNs were designed and manufactured by Sigma-Aldrich. Fluorescent microscopy was performed with a Nikon Eclipse TE2000-E inverted research microscope and MetaMorph ® software. Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO USA).

ZFN Mechanism and Donor Design

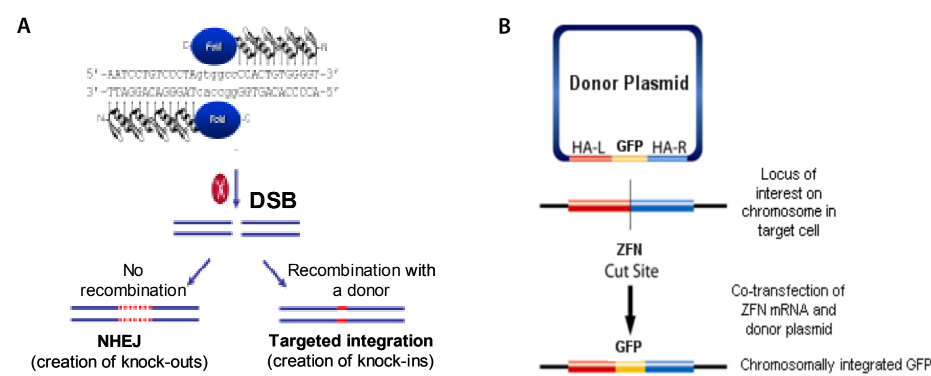


Figure 1: ZFN targeting mechanism. A. ZFNs bind to the target site. Then the FokI endonuclease domain dimerizes and makes a double strand break (DSB) between the binding sites. DSBs are repaired by either an error-prone NHEJ pathway or high-fidelity homologous recombination. NHEJ introduces deletions 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	Humanen Chromosome Number	Terminus	Distance between ZFN cut site and splice site (bp)	Initial GFP Integration Efficiency (U-2 OS Cells)	Relative Gene Expression Levels in the NCI60 Cell Lines[1]
NM_006082 (**TUBA1B**, α-tubulin 1b)	Microtubule	12	N	7	8.0%	11.91
NM_001101 (**ACTB**, β-actin)	Actin Stress Fibers	7	N	42	13.0%	13.82
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%	9.46
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%	12.85

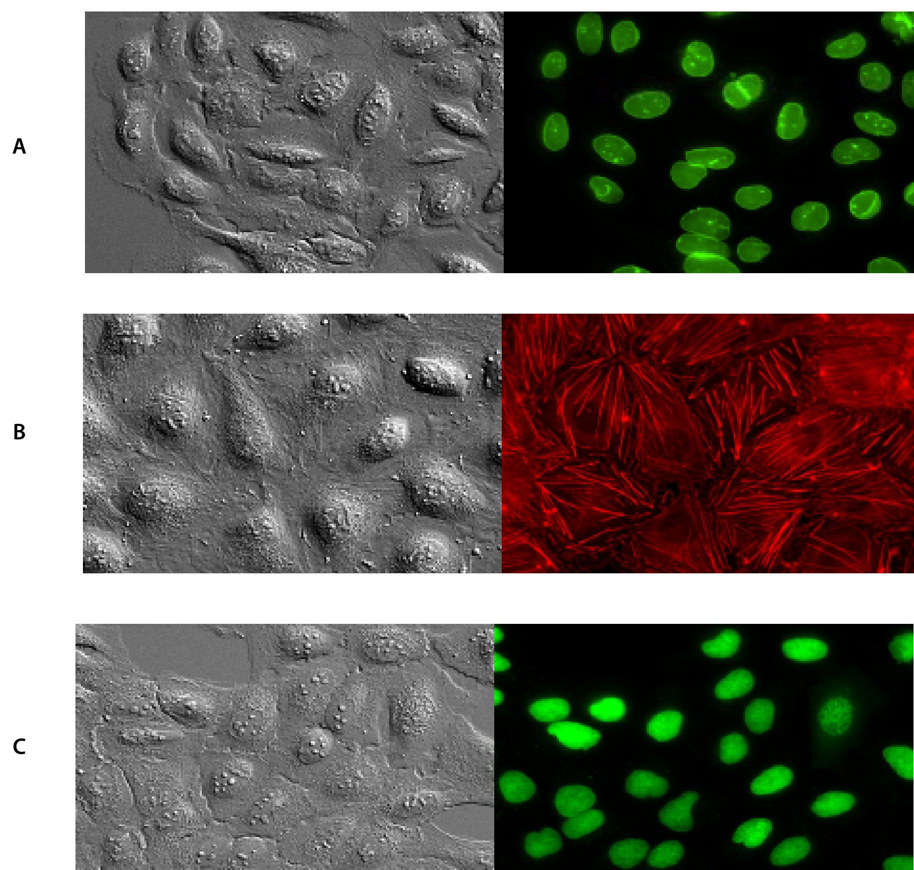
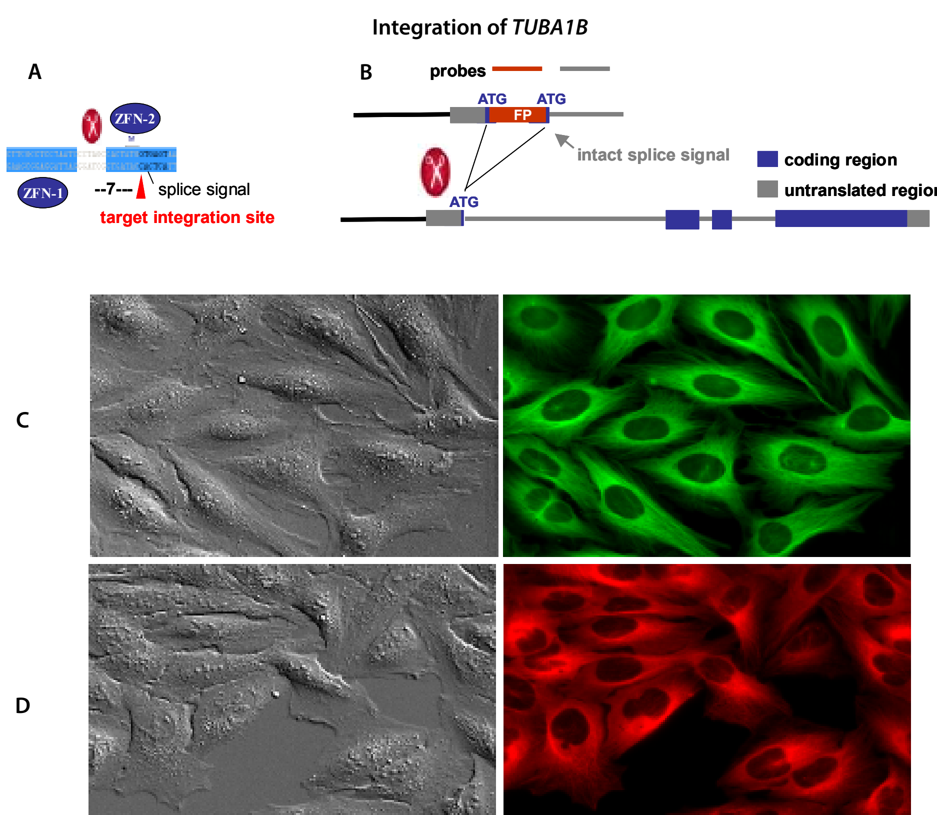


Figure 2. Successfully Tagged Loci. A. GFP integrated into the LMNB1 locus in U-2 OS cells depicting the nuclear envelope. **B.** RFP integrated into the ACTB locus in U-2 OS cells highlighting actin filaments **C.** GFP integrated into the HMGA1 locus in U-2 OS cells localized in the nucleus.

Integration of *TUBA1B*



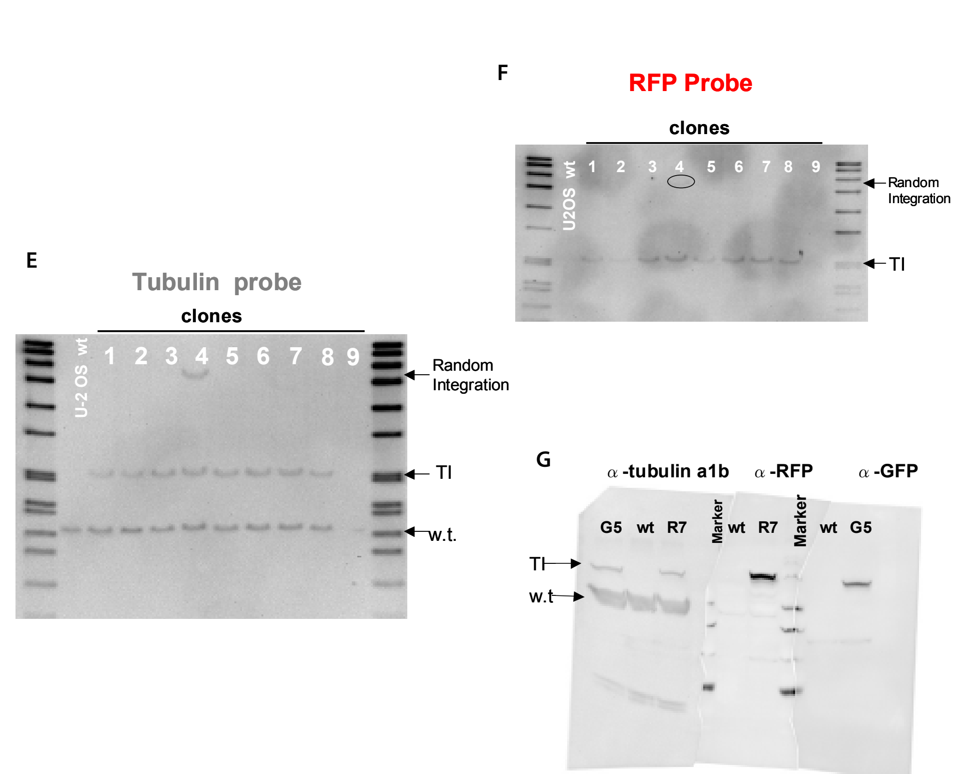


Figure 3: Targeted Integration of *TUBA1B* in U-2 OS Cells. A. Schematic of the *TUBA1B* CompoZr™ ZFN binding sites/ZFN cut site with respect to the targeted integration site. **B.** Schematic of the *TUBA1B* loci and donor(s) including location of DIG-labeled probes used in the Southern blots. DIC & fluorescent microscopy images of endogenously labeled *TUBA1B* with GFP **(C.)** and RFP **(D.)** in U-2 OS cells. Southern hybridizations were performed on DNA isolated from wild type U-2 OS cells and nine single cell clones positive for red tubulin fluorescence **(E. & F.)**. Genomic DNAs were digested with Pst I overnight. **(E.)** 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. **(F).** Using the RFP probe, hybridization to wild type U2OS DNA did not occur. However, the RFP probe did hybridize to the DNA from the RFP-*TUBA1B* clones. Out of the 9 *TUBA1B*-RFP clones, only 1 clone (#4) exhibited random integration. **G.** Western hybridizations were performed on total proteins isolated from wild type, GFP-*TUBA1B* and RFP-*TUBA1B* U-2 OS cells. Anti-*TUBA1B* indicates wild type and integrated proteins being produced in both modified cell lines. Anti-GFP and Anti-RFP indicate that fluorescent protein is being expressed in each respective cell line.

Trait Stacking

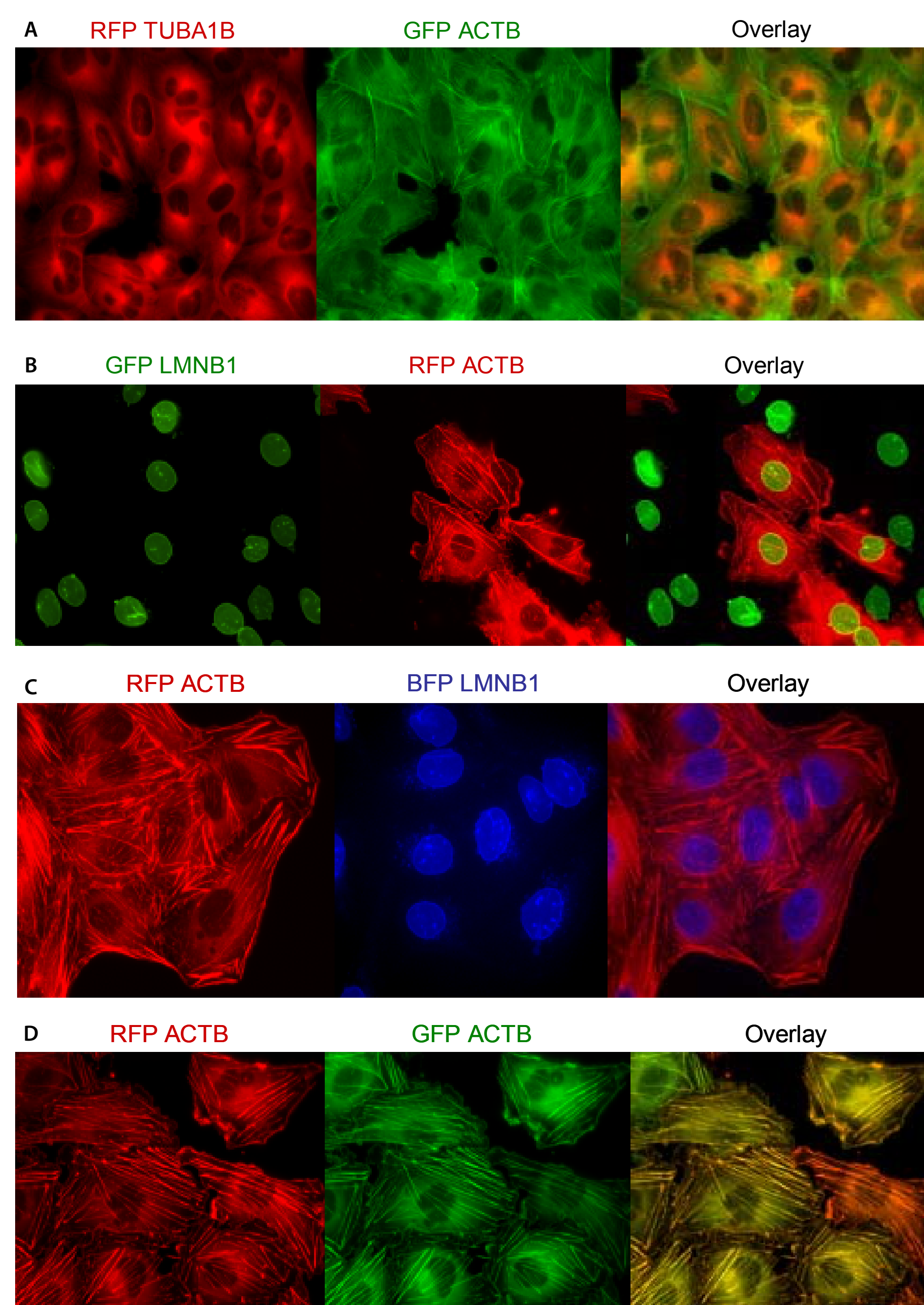


Figure 4: Trait Stacking A. A RFP *TUBA1B* expressing cell line was modified with ZFNs to also express GFP ACTB. **B.** A GFP LMNB1 expressing cell line was modified with ZFNs to also express RFP ACTB. **C.** A RFP ACTB expressing cell line was modified with ZFNs to also express BFP LMNB1. **D.** The 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.

Compound Screening

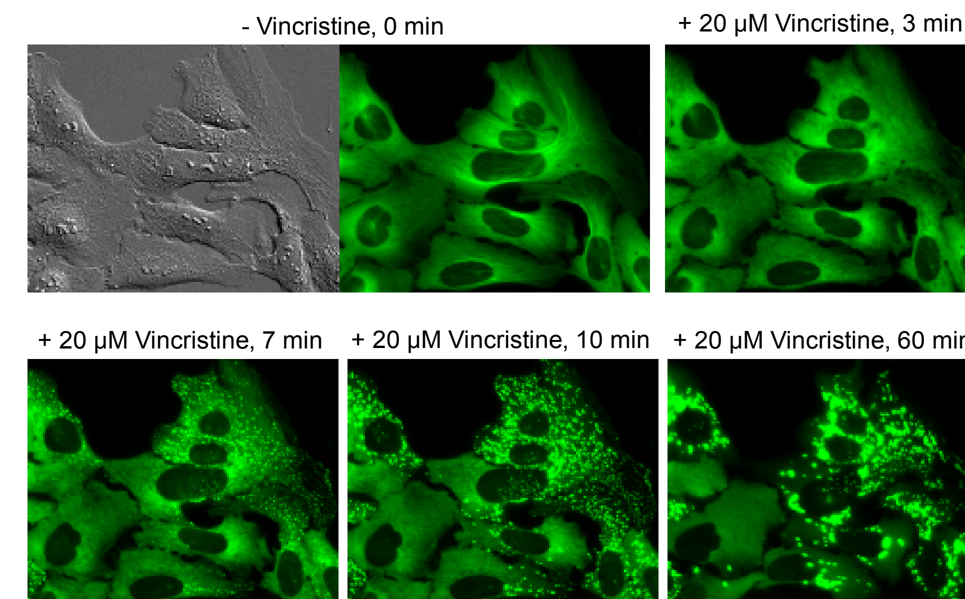


Figure 5: Vincristine time course. Vincristine is a mitotic inhibitor used in cancer chemotherapy. Its mode of action is to bind to tubulin dimers thereby inhibiting the assembly of microtubule structures.[2] GFP tagged *TUBA1B* U-2 OS cells were exposed to 20 µM Vincristine for sixty minutes. As time progresses, tubulin is repolymerized into a crystalline structure.

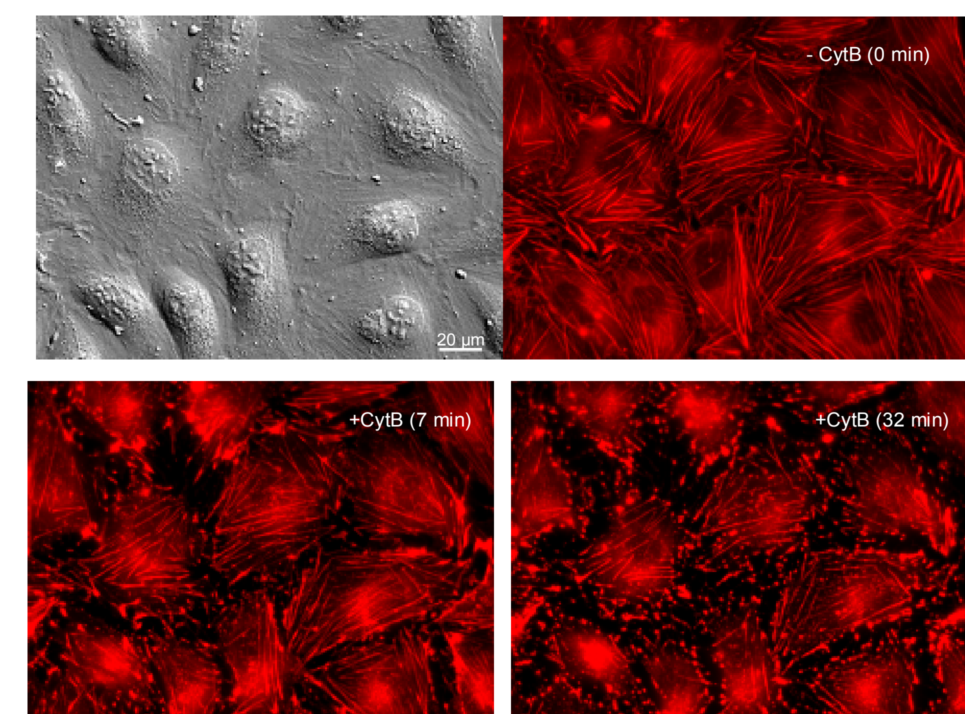


Figure 6: Cytochalasin B Time Course. Cytochalasin B is a mycotoxin. It blocks the formation of contractile microfilaments thus inhibiting cytoplasmic division[3]. By blocking monomer addition, actin filaments are shortened. RFP tagged ACTB U-2 OS cells were exposed to 21 µM Cytochalasin B. Over time, shortening of actin filaments can be observed.

Discussion/Conclusion

Until now, fluorescent labeling of proteins relied on external promoters and techniques requiring cell fixation. With ZFNs it is now possible to create stable integration of a reporter gene into a genome. Unlike fusion proteins generated with an external promoter, the fusion proteins created using the ZFNs are expressed at a physiologically relevant level in the cell. The fusion protein can be observed through out the cell's life cycle.

This work demonstrates successful tagging of four individual loci : 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). Also demonstrated are the labeling of two different genes in the same cell line as well as two different alleles of the same gene. Future work includes the study of cellular processes, compound screening, and cell-based assay development.

References/Endnotes

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Product Offerings

- Osteosarcoma Cell Line with GFP-tagged α-tubulin 1b (CLL1031)
- Osteosarcoma Cell Line with GFP-tagged β-actin (CLL1032)
- Osteosarcoma Cell Line with GFP-tagged LaminB1 (CLL1033)
- Osteosarcoma Cell Line with RFP-tagged α-tubulin 1b (CLL1034)
- Osteosarcoma Cell Line with RFP-tagged β-actin (CLL1035)

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