Published in final edited form as:

J Appl Toxicol. 2016 April ; 36(4): 568–578. doi:10.1002/jat.3255.

Distribution of Single-Wall Carbon Nanotubes in the *Xenopus laevis* Embryo after Microinjection

Brian D. Holt^{a,†}, Joseph H. Shawky^{b,†}, Kris Noel Dahl^{c,d}, Lance A. Davidson^{b,*}, and Mohammad F. Islam^{a,*}

^aDepartment of Materials Science and Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA

^bDepartment of Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

^cDepartment of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA

^dDepartment of Chemical Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA

Abstract

Single-wall carbon nanotubes (SWCNTs) are advanced materials with the potential for a myriad of diverse applications, including biological technologies and largescale usage with the potential for environmental impacts. SWCNTs have been exposed to developing organisms to determine their effects on embryogenesis, and results have been inconsistent arising, in part, from differing material quality, dispersion status, material size, impurity from catalysts, and stability. For this study, we utilized highly purified SWCNT samples with short, uniform lengths (145 ± 17 nm) well dispersed in solution. To test high exposure doses, we microinjected $> 500 \mu\text{g mL}^{-1}$ SWCNT concentrations into the well-established embryogenesis model, *Xenopus laevis*, and determined embryo compatibility and sub-cellular localization during development. SWCNTs localized within cellular progeny of the microinjected cells, but heterogeneously distributed throughout the target-injected tissue. Co-registering unique Raman spectral intensity of SWCNTs with images of fluorescently labelled sub-cellular compartments demonstrated that even at the regions of highest SWCNT concentration, there were no gross alterations to sub-cellular microstructures, including filamentous actin, endoplasmic reticulum and vesicles. Furthermore, SWCNTs did not aggregate or localize to the perinuclear sub-cellular region. Combined, these results suggest that purified and dispersed SWCNTs are not toxic to *X. laevis* animal cap ectoderm and may be suitable candidate materials for biological applications.

Correspondence to: Mohammad F. Islam, Department of Materials Science and Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA. mohammad@cmu.edu; Lance A. Davidson, Department of Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania, USA. lad43@pitt.edu.

[†]These authors contributed equally.

Conflict of interest: The authors did not report any conflict of interest.

Supporting information: Additional supporting information may be found in the online version of this article at the publisher's website.

Keywords

Xenopus laevis; carbon nanotube; Raman spectroscopy; nanoparticle toxicity; development; bovine serum albumin; actin

Introduction

Single-wall carbon nanotubes (SWCNTs) possess tremendous potential for new technologies due to their desirable mechanical, electrical and optical properties (Dresselhaus *et al.* 1996); these properties also offer unique advantages for biological applications (Liu *et al.* 2009). Because of the potential for developing organisms to be exposed to SWCNTs either intentionally, *e.g.*, using SWCNTs to image and track cells during embryogenesis, or unintentionally, *e.g.*, environmental accumulation of SWCNTs, understanding SWCNT-embryo interactions is crucial for developing SWCNT-based biotechnologies and for minimizing SWCNT ecotoxicity.

Embryogenesis is a critical period of highly coordinated, sophisticated cellular processes, when alterations to the embryonic niche from exogenous molecules may overwhelm inherent embryo defenses and result in developmental defects and/or embryonic lethality (Hamdoun *et al.* 2007). While substantial effort has been dedicated to studying the implications of numerous nanostructured carbon materials (Campagnolo *et al.* 2012; Giannaccini *et al.* 2014) including SWCNTs (Jackson *et al.* 2013) on development, little focus has been placed on (a) pure, unmodified SWCNT preparations that would be used for biotechnological applications and (b) real-space imaging of SWCNTs co-registered with sub-embryo and sub-cellular structures.

The *Xenopus laevis* (*X. laevis*) model is widely used for studying development due to its numerous advantages (Sive *et al.* 2000). For instance, amphibians are highly sensitive to environmental contaminants and are used as standard models for toxicity screening (Bridges *et al.* 2002). FETAX (Frog Embryo Teratogenesis Assay – *Xenopus*) is a standardized assay developed by the U.S.A. Environmental Protection Agency (Bacchetta *et al.* 2012a; Delgado *et al.* 2013; Dumont *et al.* 1983; Hutchison *et al.* 2014). Some other advantages of the *Xenopus* embryo model are that fertilization occurs *ex vivo* at room temperature, the fertilized ovum is about a millimeter in diameter, the embryo develops rapidly from fertilization to gastrulation in approximately 9 hours, and the progressive stages of development can be imaged with a simple stereomicroscope (Sive *et al.* 2000). *X. laevis* embryos can be indirectly exposed to materials dispersed in media or directly exposed to materials *via* microinjection into the embryo. Different tissues can be excised from the embryo using microsurgical manipulations and remain viable which allows for imaging of different surfaces and tissues within the embryo.

SWCNTs possess specific, intense Raman scattering modes, including the G-band at $\sim 1591\text{ cm}^{-1}$ and radial breathing modes (RBMs) at $\sim 100 - 500\text{ cm}^{-1}$ due to their tubular structure of sp^2 hybridized carbon (Dresselhaus *et al.* 2005). SWCNTs' specific Raman scattering can be confocally imaged with high spatial resolution within biological systems. Raman imaging of SWCNTs offers numerous advantages over other imaging modalities such as bright field

absorbance imaging and transmission electron microscopy (TEM). Raman imaging does not require labeling or sample preparation that could alter the specimen, possesses high sensitivity and specificity, has diffraction-limited spatial resolution in the three spatial dimensions and allows for co-registration to fluorescently labeled, specific components.

To determine embryo compatibility and cellular localization of SWCNTs, we microinjected highly purified (~ 0.3 wt.% metallic impurities and < 5 wt.% carbonaceous impurities) (Islam *et al.* 2005; Johnston *et al.* 2005), short and length fractionated (145 ± 17 nm long) (Holt *et al.* 2010), primarily individually dispersed (*i.e.*, not in bundles) with bovine serum albumin (BSA) (Holt *et al.* 2011; Holt *et al.* 2012a) SWCNTs into *X. laevis* embryos at the four-cell stage. We analyzed SWCNT-mediated embryo toxicity, imaged the overall distribution of SWCNTs within developing embryos and their sub-cellular localization. SWCNTs did not induce deleterious effects, even at high exposure doses of ~ 3 ng per embryo. SWCNTs remained individually dispersed, were distributed throughout the embryo and ultimately were located in the peri-nuclear regions of cells that had internalized the injection volume without altering sub-cellular compartments. These results suggest that well-prepared SWCNTs are not toxic to *X. laevis* animal cap ectoderm and may be candidate materials for cell biological applications.

Materials and methods

Nanotube dispersions

Single-wall carbon nanotubes were highly purified, length fractionated and well-dispersed as described previously. Briefly, high pressure carbon monoxide conversion synthesis (HiPCO) SWCNTs were purified *via* wet air burn in the presence of H_2O_2 , acid reflux, magnetic fractionation and annealing (Islam *et al.* 2005; Johnston *et al.* 2005). The purified SWCNTs contained < 5 wt.% carbonaceous and ~ 0.3 wt.% metallic impurities (Islam *et al.* 2005) without a change in SWCNT properties (Johnston *et al.* 2005). SWCNTs were dispersed with deoxycholic acid (Islam *et al.* 2003) and length fractionated by density gradient ultracentrifugation (Arnold *et al.* 2006). SWCNTs with lengths of 145 ± 17 nm were selected and the dispersing agent burned off (Holt *et al.* 2010).

SWCNTs are hydrophobic and interact with each other with an energy of $\sim 40 k_B T/nm$ (Girifalco *et al.* 2000; Hough *et al.* 2004); thus, unmodified SWCNTs are highly bundled in aqueous conditions. Exfoliation into individually dispersed SWCNTs for applications was achieved by sonication of SWCNTs in the presence of a dispersing agent in water, as previously described: 1.0 wt.% bovine serum albumin (BSA) and 0.1 wt.% SWCNTs, subjected to 2 h of probe-tip sonication (Fisher Scientific, Sonic Dismembrator Model 100, 3 mm probe tip) at 6 W (Holt *et al.* 2011; Holt *et al.* 2012a). Centrifugation at $21,000 \times g$ for 7 min (Holt *et al.* 2011) was performed to pellet remaining small bundles. Supernatants were decanted and the concentration of SWCNTs was determined by a known absorbance coefficient at 930 nm (Boyer *et al.* 2013; Holt *et al.* 2012a; Holt *et al.* 2012b; Holt *et al.* 2010). Dispersions were sterilized by UV lamp (UVP Black-Ray, 100 W long-wave ultraviolet lamp, Model B 100 AP/R; UVP, LLC, Upland, CA, USA) for 1 h. Previous description of ultraviolet-visible-near-infrared absorbance, fluorescence and Raman spectroscopies of the BSA-stabilized SWCNTs showing highly resolved van Hove peaks are

included elsewhere (Holt *et al.* 2011; Holt *et al.* 2012a) and confirm that the supernatants of SWCNTs prepared in this manner contain mostly individualized SWCNTs with some very small bundles (Bachilo *et al.* 2002; Dresselhaus *et al.* 1996; Kataura *et al.* 1999; O'Connell *et al.* 2002).

Embryos and nanotube microinjection

X. laevis embryos were obtained by standard methods (Kay 1991), fertilized *in vitro*, dejellied in 2 wt.% cysteine and cultured in (1/3)× Modified Barth's Saline (MBS; 29 mM NaCl, 0.27 mM MgSO₄, 0.8 mM NaHCO₃, 0.33 mM KCl, 0.11 mM Ca(NO₃)₂, 0.14 mM CaCl₂ and 3.3 mM HEPES) (Sive *et al.* 2000) at 22 °C. Embryos were microinjected 4× in the animal region at the four-cell stage with a total volume of 2, 4, 8, 16 or 32 nL of 1.0 wt.% BSA suspension or 4 nL of BSA-stabilized SWCNTs at a concentration of 700 µg mL⁻¹ and cultured to tailbud stage (stage 29) or until death. Staging of embryos was performed according to the table of Nieuwkoop and Faber (Nieuwkoop 1967). Embryos were kept in 3% Ficoll solution made in (1/3)× MBS during injection then transferred back to regular (1/3)× MBS after healing (~30 min).

To isolate animal cap explants, early gastrula (stage 10 – 10.5) embryos were selected and transferred to culture media, Danilchik's For Amy (DFA), containing 53 mM NaCl, 5 mM Na₂CO₃, 4.5 mM K Gluconate, 32 mM Na Gluconate, 1 mM MgSO₄, 1 mM CaCl₂ and 1 g of BSA per 1 l, and then buffered to pH 3 with 1 M bicine, for microsurgery. For single cell experiments, animal caps were dissociated by incubation in Ca²⁺/Mg²⁺ free DFA for 5 – 10 min and returned back to normal DFA to allow adhesion to the fibronectin-coated substrate (Roche, Mannheim, Germany; 25µg mL⁻¹) before fixation.

Confocal fluorescence imaging

Dissociated cells were fixed in 3.7% formaldehyde / 0.25% glutaraldehyde in phosphate buffered saline (PBS) for 10 – 15 min. F-actin was visualized by incubating fixed cells for 1 – 2 h with BODIPY-FL-Phalloidin (2.5:1000) in PBST (0.1% Triton X). Following phalloidin staining, cells were dehydrated in isopropanol and cleared in Murray's clear (2:1 benzyl benzoate:benzyl alcohol). Single optical sections and Z-stacks of fixed samples were collected using a 63× 1.4 numerical aperture (NA) oil-immersion objective mounted on an inverted compound microscope (DMI6000, Leica Microsystems) equipped with a confocal laser scan head (SP5, Leica Microsystems) using Leica Application Suite Advanced Fluorescence (LASAF, Leica Microsystems) software. Maximum projections and reslicing of z-stacks were carried out with ImageJ (v. 1.38, Wayne Rasband, NIH). Three animal cap explants were subjected to confocal fluorescence imaging and the same explants were separately imaged with confocal Raman spectroscopy. For dissociated cells, at least 40 cells were imaged with confocal fluorescence imaging and then separately imaged with Raman spectroscopy.

Widefield fluorescence imaging

Dissociated cells were fixed in 3.7% formaldehyde / 0.25% glutaraldehyde in PBS for 10 – 15 minutes, permeabilized for 5 min with 0.2% wt.% Triton X-100 and labeled with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) at 0.25 µg mL⁻¹, Oregon Green® 488

phalloidin at 0.165 μM and FM® 4-64 at 25 μM for 30 min (all fluorophores were from Life Technologies). Widefield fluorescence imaging was performed on a Leica DMI 4000B fluorescence and light microscope with a 100 $\times$ 1.4 NA oil-immersion objective. Over 10 cells were fluorescently imaged and the same cells were separately imaged with Raman spectroscopy.

Raman spectroscopy and imaging

Confocal Raman spectroscopy and imaging was performed on an inverted Raman microscope (inVia Raman microscope, Renishaw) with a 100 $\times$, 1.4 NA oil-immersion objective and a 785 nm (1.58 eV) laser of ~ 10 mW at the sample plane. Phase contrast imaging was performed on the same microscope including the use of a 0.9 NA condenser. Control of Raman mapping parameters, including 3D position, was performed using WiRE 3.4 software (Renishaw). Different Raman maps were acquired with different step sizes, ranging from 0.5 – 20 μm , depending on the zoom level. Large fields of view were generated by automatically concatenating multiple fields of view together in WiRE software.

The presence of SWCNT G-band in Raman spectra at $\sim 1591\text{ cm}^{-1}$ indicates sp^2 hybridized carbon structures (Dresselhaus *et al.* 2005), and we have previously shown that its intensity can be used to determine SWCNT concentration (Boyer *et al.* 2013; Holt *et al.* 2011; Holt *et al.* 2012a; Holt *et al.* 2012b). The intensity ratio $I_D:I_G$ between the SWCNT D-band, which characterizes the sp^3 -hybridized carbon, and the G-band indicates damage or structural defects in SWCNTs. For SWCNTs prepared *via* HiPCO synthesis, their diameters are ~ 1 nm, and when subjected to Raman spectroscopy with a 785 nm (1.58 eV) laser, RBMs $< 250\text{ cm}^{-1}$ arise from individualized, that is not bundled, SWCNTs whereas RBMs $> 250\text{ cm}^{-1}$ arise from bundles of SWCNTs (Dresselhaus *et al.* 2005; Heller *et al.* 2004). This is because the SWCNT (10,2) chirality comes into resonance with the 785 nm laser when it is within bundles of SWCNTs but not when it remains individually dispersed (Heller *et al.* 2004). Also, real space SWCNT near-infrared (NIR) fluorescence was also detected in the Raman spectra from $\sim 2100 - 3000\text{ cm}^{-1}$ which only arises from individualized SWCNTs (O'Connell *et al.* 2002). We have previously used these Raman spectral features to determine SWCNT dispersion state within cells (Holt *et al.* 2012a). A Raman spectrum of the initial SWCNT dispersion, fluorescence spectroscopy of SWCNTs, a summary of the SWCNT diameters, and further discussion on using the RBMs to determine SWCNT bundling is included in Supporting Information, Fig. S1.

Therefore, RBMs can be used to probe local SWCNT dispersion status in cells (Holt *et al.* 2012a). For mapping experiments, spectra were acquired at Raman shifts of 1188 – 1696 and 197 – 802 cm^{-1} for G-band and RBM scans, respectfully. Analysis of Raman mapping experiments was performed using in-house MATLAB® (The MathWorks, Inc.) code: G-band and RBM signal above baseline (having a signal-to-noise ratio > 3) was used to color-code intensity maps. For G-band, the code was especially designed to accurately determine G-band signal while differentiating it from other signals from the explants at $\sim 1580\text{ cm}^{-1}$ and $\sim 1600\text{ cm}^{-1}$, in addition to determining non-uniform backgrounds.

Fluorescence/SWCNT overlays

Confocal fluorescence Z-stacks and widefield fluorescence images were acquired on separate imaging systems than Raman maps. To co-register fluorescence and Raman signals, the same field of view was imaged on both systems. Then, as indicated, confocal or widefield fluorescence images were overlaid with confocal Raman images by matching the fluorescence signals (confocal) or phase image (widefield) with the phase image acquired on the Raman system. Images were adjusted to be the same size and overlays were generated using LASAF software.

For Z-stack overlays of confocal fluorescence and confocal Raman, more processing needed to be performed as Raman Z-stacks were sub-sampled compared to fluorescence: Raman Z-step size was 2 μm was fluorescence was 0.2 μm . Therefore, Raman Z-stacks were interpolated (spline interpolation in MATLAB) to generate the same number of images as fluorescence. Alignment of Z-positions was based on where confocal F-actin intensity began and where phase contrast (on the Raman system) just came into focus. While theoretically lipid or other biological molecule peaks could be used to determine the Z-position on the Raman system, practically there was not enough signal to discriminate these peaks. While phase contrast is not a confocal imaging modality, similar processing was performed to it to create Z-stack, and phase intensity indeed varied with Z-position.

Results

Single-wall carbon nanotubes are not toxic

To maximize direct exposure of SWCNTs to *X. laevis*, we microinjected suspensions of SWCNTs into the animal hemisphere of all four blastomeres at the four-cell stage. To determine appropriate injection volumes of SWCNT suspensions, we first microinjected a series of volumes (2, 4, 8, 16 and 32 nL) of the dispersing agent BSA at its concentration used in the dispersion procedure, 1.0 wt.%, to determine the non-toxic, upper-limit injection volume. Microinjection of 4 nL of 1.0 wt.% BSA suspension resulted in normal development to tailbud stage (stage 29) while volumes > 4 nL were toxic and inhibited proper development (Table 1). Embryos injected with 8 nL developed defects including stalled gastrulation and/or shortened axes; injections of 16 nL induced early lethal defects including failure of blastomeres to cleave properly. Hence, all subsequent experiments were performed using an injection volume of 4 nL.

To determine whether microinjections of SWCNTs into the animal hemisphere of the *X. laevis* embryo induced any developmental defects, we injected the non-toxic upper-limit volume of 4 nL of highly concentrated ($700 \mu\text{g mL}^{-1}$) SWCNTs into prospective animal cap tissue cells at the 4-cell stage. The animal cap is a dual layer tissue composed of deep mesenchymal cells with an overlying epithelium that is the precursor to the skin and nervous system of the tadpole. This tissue can be precisely targeted by microinjection and is easily visualized *in vivo* or *in vitro* by microsurgically excising it from the rest of the embryo followed by culturing on fibronectin. Tissue movements in the animal cap, including radial intercalation and outward spreading are well characterized. Therefore, variations from native behaviors due to exogenous influences such as SWCNT injection would be easily detected.

Injections of SWCNTs into these tissues yielded no deleterious effects on development and were nontoxic (Table 1). Embryos successfully developed through tailbud stage (stage 29), demonstrating that microinjection of ~ 3 ng of SWCNTs into embryos did not affect *X. laevis* development.

Nanotubes are distributed throughout the animal cap ectoderm

To determine the localization of SWCNTs within developing embryos, microinjected *X. laevis* embryos were allowed to develop to gastrula stage (stage 10 – 10.5) when animal cap ectoderm tissues were microsurgically isolated, fixed and mounted for imaging. Animal cap ectoderm was imaged using high resolution phase contrast microscopy, and the spatial distribution and local concentration of SWCNTs were imaged using Raman spectroscopy. We imaged 10 separate animal caps, and the results were consistent between samples. Co-registration of embryo features with SWCNT signal revealed that SWCNTs were heterogeneously distributed throughout the animal cap (Fig. 1A). Some regions possessed high SWCNT concentrations ($> 100 \mu\text{g mL}^{-1}$) which were approximately similar to the concentration of the injection ($700 \mu\text{g mL}^{-1}$), while many regions throughout the animal cap tissue contained less concentrated SWCNTs ($< 25 \mu\text{g mL}^{-1}$). Higher spatial resolution heat maps of the distribution and concentration of SWCNTs revealed centers of highly concentrated SWCNTs away from which concentration decreased (Fig. 1B). There were no observable alterations to embryonic tissue structures in regions with SWCNTs, even for those with high concentrations.

The dispersion state of SWCNTs, or in other words if SWCNTs remained individually dispersed or became bundled, is important for the physical properties of SWCNTs (Dresselhaus *et al.* 1996; Hersam 2008; Jariwala *et al.* 2013; O'Connell *et al.* 2002) and biological interfacial properties (Battigelli *et al.* 2013; Bilalis *et al.* 2014; Heister *et al.* 2013). Raman spectra of SWCNTs were used to determine the dispersion state of SWCNTs. Spectra contained numerous peaks arising from the animal cap itself; nonetheless, peaks that are specific for SWCNTs were readily detected, including the G-band at $\sim 1591 \text{ cm}^{-1}$ and RBMs between $200 - 275 \text{ cm}^{-1}$ (Fig. 1C). The relatively high ratio of RBM intensity $< 250 \text{ cm}^{-1}$ to RBM intensity $> 250 \text{ cm}^{-1}$, along with the presence of NIR fluorescence of SWCNTs, manifesting as a broad peak within $2100 - 3000 \text{ cm}^{-1}$, confirmed the presence of individually dispersed SWCNTs and that SWCNTs did not aggregate into large bundles.

Nanotubes are distributed in three-dimensions

To determine the three dimensional (3D) spatial distribution of SWCNTs within *X. laevis* embryos and whether microinjection of SWCNTs induced disruption of the earliest manifestation of multi-laminate tissue structure, we performed confocal Raman imaging of the intensity of the G-band of SWCNTs co-registered with confocal fluorescence imaging of rhodamine phalloidin that labeled filamentous-actin (F-actin) structures. We imaged F-actin because it is a highly prevalent protein, plays crucial roles in numerous cellular processes including division, motility, endocytosis *etc.* (Pollard *et al.* 2008) and could be used to delineate individual cells.

Confocal maximum Z-projected stacks of animal caps microinjected with SWCNTs showed normal F-actin structures (Fig. 2) and were consistent between samples. SWCNTs were distributed throughout the animal cap ectoderm in 3D, and SWCNTs neither co-localized with F-actin structures nor induced alterations to F-actin structures.

Nanotubes are localized within cells that internalized the injection volume

To further investigate how SWCNTs were distributed throughout the *X. laevis* animal cap, we co-injected SWCNTs with 0.5 ng of rhodamine-conjugated 10 kDa dextran per blastomere at the four cell stage. Labeled dextrans microinjected into embryos do not transfer from cell to cell and localize to the cytoplasm of injected cells where they can be used for lineage tracing (Gimlich *et al.* 1985). Therefore, the co-distribution of SWCNTs and fluorescently-labeled dextrans could demonstrate whether SWCNTs remained localized in the cytoplasm of injected cells.

Due to the opacity of *X. laevis* embryos and the need to accurately co-register Raman images with fluorescence images, we performed single cell layer imaging rather than imaging the entire, intact animal cap explants. To enable single cell imaging, we dissociated animal caps, allowed the individualized cells to adhere to the substrate, then fixed and labeled all cells with BODIPY® FL phalloidin into label F-actin.

Confocal maximum Z-projections of F-actin and dextrans clearly indicated that some cells inherited fluorescently labeled dextrans while other cells did not (absence of fluorescence from dextrans but presence of fluorescence from F-actin structures) (Fig. 3 and Supporting Information, Fig. S2). Confocal Raman imaging of the same field of view reveal SWCNTs in cells that are fluorescently labeled with dextrans. These results were consistent across all samples. Therefore, SWCNTs were incorporated into and distributed throughout the progeny of the cells exposed to the injection bolus, like the fluorescently labeled dextrans.

To determine the dispersion state of SWCNTs, we acquired Raman spectra of the RBMs of SWCNTs and plotted heat maps of the intensity of RBMs that arise from individualized SWCNTs (Fig. 3 and Supporting Information, Fig. S2). Like the injected dispersion, SWCNTs possessed significant RBM intensity from individualized SWCNTs, and a representative Raman spectrum clearly showed high intensity of RBMs from individually dispersed SWCNTs (RBM intensity $< 250 \text{ cm}^{-1}$) along with broad NIR fluorescence of SWCNTs ($\sim 2100 - \sim 3000 \text{ cm}^{-1}$), confirming that SWCNTs remained individually dispersed and were not bundled. Additionally, the $I_D:I_G$ was 0.05 ± 0.02 for the intracellular SWCNTs, which is similar to that of SWCNTs before microinjection of 0.03 ± 0.02 .

Nanotubes are localized in peri-nuclear regions and do not alter sub-cellular compartments

To determine the sub-cellular localization of SWCNTs and potential alterations to sub-cellular compartments, we fluorescently labeled important cellular compartments and imaged individual cells. To do so, we dissociated animal cap ectoderm tissues, allowed the individualized cells to adhere to the fibronectin-coated substrate, then fixed and labeled the cells with DAPI to label DNA, Oregon Green phalloidin to label F-actin and FM 4-64 to label endoplasmic reticulum (ER) and vesicles.

Fluorescence imaging of cellular compartments consistently revealed no gross change to their localization or morphology (Fig. 4 and Supporting Information, Fig. S3). Co-registering fluorescence images of cellular components and the Raman image of SWCNT concentration revealed high concentrations of SWCNTs that were not co-localized with sub-cellular structures but were primarily located in the peri-nuclear regions. SWCNTs did not alter the localization of the sub-cellular compartments, even though some regions had concentrations $> 100 \mu\text{g mL}^{-1}$. For selected regions of high SWCNT concentrations, higher spatial resolution (smaller step size between spectral acquisitions) Raman imaging further confirmed that SWCNTs were located in the peri-nuclear regions without alterations to cellular compartments (insets of Fig. 4).

To obtain high spatial resolution images of SWCNT dispersion status, Raman spectra of the RBMs of SWCNTs were acquired. Imaging revealed substantial intensity of RBMs $< 250 \text{ cm}^{-1}$, demonstrating that SWCNTs remained well dispersed (*i.e.*, did not aggregate) within cells although their local concentration remained high (Supporting Information, Fig. S3).

Discussion

Singl-wall carbon nanotubes are not toxic

Several studies have found embryonic defects induced by nanostructured carbon materials, including nanodiamonds (Marcon *et al.* 2010; Vankayala *et al.* 2014; Wierzbicki *et al.* 2013), paracrystalline carbon powder (Bacchetta *et al.* 2012b; Cheng *et al.* 2007; Jackson *et al.* 2012; Pietroiusti *et al.* 2011), fullerenes (Kim *et al.* 2012; Murugesan *et al.* 2007; Vankayala *et al.* 2014; Wierzbicki *et al.* 2013), graphite (Chen *et al.* 2012; Murugesan *et al.* 2007; Wierzbicki *et al.* 2013), multi-wall carbon nanotubes (Chen *et al.* 2012; Cheng *et al.* 2009; Cheng *et al.* 2012; Hougaard *et al.* 2013; Huang *et al.* 2014; Kim *et al.* 2012; Liu *et al.* 2014; Murugesan *et al.* 2007; Nouara *et al.* 2013; Wierzbicki *et al.* 2013) and SWCNTs (Campagnolo *et al.* 2013; Chen *et al.* 2013; Cheng *et al.* 2007; Cheng *et al.* 2011; Huang *et al.* 2014; Leeuw *et al.* 2007; Pietroiusti *et al.* 2011; Roman *et al.* 2013). However, relatively few studies have used the *X. laevis* model, even though it is especially advantageous to examine potential cytotoxic effects of nanomaterials because it is highly sensitive to environmental contaminants and provides an *in vivo* environment in which cells are undergoing cell division, differentiation, migration, and vesicular transport all while maintaining adhesions with neighboring cells and extracellular matrix (Bridges *et al.* 2002).

Prior studies using *X. laevis* embryos found nanostructured carbon materials could be toxic. For example, microinjected, nearly spherical $\sim 4 \text{ nm}$ diameter nanodiamonds had toxicity that was highly dependent on nanodiamond interfacial properties, as carboxyl functionalization resulted in embryotoxicity and teratogenicity but hydroxyl functionalization was not toxic, even for a 10 nL microinjection of 2 mg mL^{-1} nanodiamonds (Marcon *et al.* 2010). Other studies exposed *X. laevis* to nanostructured carbon materials in suspension. A series of studies from the same group determined that undispersed carbon nanotubes were cytotoxic but not genotoxic (Mouchet *et al.* 2011; Mouchet *et al.* 2010; Mouchet *et al.* 2008; Saria *et al.* 2014). Double-wall carbon nanotubes accumulated in the gills and intestines, resulting in growth inhibition and embryotoxicity but minimal genotoxicity (Mouchet *et al.* 2011; Mouchet *et al.* 2008). Similar results were

obtained for multi-wall carbon nanotubes, as they inhibited growth, increased oxidative stress and induced DNA damage (Mouchet *et al.* 2010; Saria *et al.* 2014). Other groups also demonstrated that multi-wall carbon nanotubes were embryotoxic at exposure doses of $50 \mu\text{g mL}^{-1}$ but were not genotoxic (Bourdiol *et al.* 2013). Amorphous carbon spheres of diameters of $28.5 \pm 14.3 \text{ nm}$ also led to toxicity at an exposure dose of $100 \mu\text{g mL}^{-1}$, and the nanoparticles preferentially accumulated in the stomach and gut (Bacchetta *et al.* 2012b).

Many of these studies utilized materials that were either not highly purified or not individually dispersed. Many nanostructured carbon materials are synthesized with toxic catalysts, and syntheses typically also produce carbonaceous impurities. Without extensive purification, material/embryo interactions may be dominated by these minority components. Further, as nanostructured carbon materials are hydrophobic, this can lead to aggregation of not well dispersed materials in biological environments, complicating material/embryo interactions. While studies of as-produced or minimally processed materials are crucial for understanding the toxicity of these materials that, for example, may be used in industrial applications, they are not directly applicable to highly purified, well-dispersed materials that, for example, may ultimately be utilized for biological applications.

Therefore, in this study we investigated ultrapure ($\sim 0.3 \text{ wt.}\%$ metal and $< 5 \text{ wt.}\%$ carbonaceous impurities), length selected ($145 \pm 17 \text{ nm}$), individually dispersed SWCNTs ($\sim 1 \text{ nm}$ diameter, individual SWCNTs) (Holt *et al.* 2011; Holt *et al.* 2012a; Holt *et al.* 2010), as this preparation has potential for use in biotechnological. SWCNTs were purified to remove virtually all toxic catalyst, length fractioned to avoid altered cellular interactions due to lengths varying over a couple orders of magnitude and individually dispersed to remove bundles that have larger diameters that could affect interactions with embryo structures and reduce SWCNT properties.

These highly processed SWCNTs possessed no toxicity to *X. laevis* embryos up to $\sim 2.8 \text{ ng}$ of SWCNTs delivered *via* 4 nL microinjection directly into their cytoplasm. 4 nL was an upper limit on injection volume because the dispersing agent control solution of $1.0 \text{ wt.}\%$ BSA caused toxicity for injections $> 4 \text{ nL}$. We attribute the toxicity of higher injection volumes to the surfactant-like nature of BSA (Curry *et al.* 1999) that could solubilize embryo proteins and membranes and the osmotic pressure of the ultrapure water that could substantially affect local osmolarity and pH, as observed previously (Dubertret *et al.* 2002). $\sim 700 \mu\text{g mL}^{-1}$ was a reasonable upper limit on SWCNT concentration since concentrations $> \sim 3 \text{ mg mL}^{-1}$ alter SWCNT state (Hough *et al.* 2006; Puech *et al.* 2011). Therefore, microinjecting $\sim 3 \text{ ng}$ of SWCNTs was an effective upper limit for our system, and at this maximum exposure dose, there was no SWCNT toxicity.

We attribute the lack of toxicity of our SWCNTs due to the extensive materials preparation. By removing virtually all metal catalyst that remained from SWCNT synthesis (Islam *et al.* 2005; Johnston *et al.* 2005), we avoided metal impurity toxicity that is unavoidable for unpurified SWCNTs (Kagan *et al.* 2006). Length selecting SWCNTs to be $145 \pm 17 \text{ nm}$ long enabled endocytosis and exocytosis (Becker *et al.* 2007). Individually dispersed SWCNTs have been shown to be more cytocompatible than bundles, enabling organism and cellular recovery from SWCNT exposure and allowing for long-term cellular labeling without

altering cellular state or function (Heller *et al.* 2005; Holt *et al.* 2012a; Jin *et al.* 2009; Jin *et al.* 2008; Kolosnjaj-Tabi *et al.* 2010; Liu *et al.* 2013; Ruggiero *et al.* 2010).

Injected nanotubes distribute throughout the targeted animal cap ectoderm

Unlike many studies that solely relied on the presence of dark regions in bright field microscopy or relatively large aggregates of SWCNTs in TEM to identify the spatial localization of SWCNTs, we utilized SWCNTs' optical property of unique Raman scattering to specifically and sensitively non-invasively image SWCNT distribution without requiring extensive sample preparation that could potentially alter SWCNT sub-embryo localization. While highly optically absorbing regions of bright field images may allow for rapid, accessible imaging, dark regions are not necessarily SWCNTs and light regions are not necessarily SWCNT-free, and these exceptions have been experimentally reported for *X. laevis* embryos (Mouchet *et al.* 2011; Mouchet *et al.* 2008). TEM of multi-wall carbon nanotubes in *X. laevis* larvae required careful staining to generate images of proper contrast and sectioning samples with an ultramicrotome which may have altered their distribution within the biological matrix (Bourdiol *et al.* 2013). Here, we acquired Raman spectra in a point-by-point manner and generated heat maps of SWCNTs' unique, intense G-band (Dresselhaus *et al.* 2005). Additionally, by utilizing a previously established calibration curve (Holt *et al.* 2012a), we were able to quantitatively determine the local concentration of SWCNTs.

Utilizing the high sensitivity, we determined that SWCNTs were distributed throughout the cells of the animal cap in a somewhat heterogeneous manner. Many SWCNT-containing regions had concentrations $\sim 10\times$ lower than the injection dose while a few regions remained highly concentrated, similar to the injection dose concentration. These results suggest that the cells that were initially injected subsequently proliferated and distributed SWCNTs throughout their daughter cells. Overall, the SWCNTs were inert and did not alter embryonic development or structures of the animal cap ectoderm.

Nanotubes are distributed in 3D

While confocal fluorescence and Raman imaging enabled the generation of 3D stacks of co-registered images, there were some limitations. Raman imaging was performed in the X–Y plane over a Z-height of 10 μm with a 2 μm Z-direction step size. Since the F-actin fluorescence was acquired with a 0.2 μm step size, the Raman and phase contrast images were interpolated to create Z-stacks of the same number of images to enable overlaying. Additionally, confocal fluorescence and confocal Raman imaging were performed using different imaging modalities; therefore, there was some inherent uncertainty in the co-registration of spatial localization between the two imaging systems. Finally, phase contrast images, which are not confocal, were acquired at each Raman Z-step and used to align the Raman spatial position with the fluorescence position. Nonetheless, we were able to generate Z-stacks that demonstrated SWCNTs' 3D localization within the animal cap ectoderm of *X. laevis* embryos.

Nanotubes are retained by cell lineages that were injected

Fluorescently labeled dextrans have been used as cellular lineage tracers, as microinjections are uniformly distributed throughout the cytoplasm of the progeny of the microinjected cells (Gimlich *et al.* 1985). Therefore, fluorescently labeled dextrans can be used to image the cellular progeny of the microinjected cells. By dissociating animal caps, we were able to accurately co-register SWCNT cellular localization with fluorescently labeled dextrans and probe sub-cellular SWCNT distribution. Raman imaging demonstrated that SWCNTs were present within cells that also contained the fluorescently labeled dextrans but were not present in progeny from cells that did not receive the microinjection of SWCNTs, as expected (Fig. 3 and Supporting Information Fig. S2). This distribution of SWCNTs is in agreement with previous results that microinjections of well dispersed quantum dots into *X. laevis* embryos were cell autonomous, that is the quantum dots were retained within the progeny of the microinjected cell (Dubertret *et al.* 2002).

As indicated for quantum dots, stability of the nanoparticle dispersion is important for cytocompatibility and ultimate use for biotechnological applications (Dubertret *et al.* 2002). We have previously shown that the BSA-dispersed SWCNTs used in this study were stable upon dilution in ultrapure water (Holt *et al.* 2011) and cell culture media (Boyer *et al.* 2013; Holt *et al.* 2012b). Therefore, SWCNTs should have remained well dispersed after injection into *X. laevis*, and indeed, Raman spectroscopy confirmed that SWCNTs remained well dispersed within *X. laevis*. Thus, we suggest that since SWCNTs remained individualized, this facilitated their distribution throughout the cellular progeny that recapitulated the distributions of the simultaneously microinjected fluorescently labeled dextrans.

Nanotubes are sub-cellularly localized in peri-nuclear regions and do not alter sub-cellular compartments

There have been reports in the literature by our group (Holt *et al.* 2015; Holt *et al.* 2012c; Holt *et al.* 2010; Shams *et al.* 2014; Yaron *et al.* 2011) and others (Dulinska-Molak *et al.* 2014; Kaiser *et al.* 2008; Rodriguez-Fernandez *et al.* 2012; Sargent *et al.* 2012; Villegas *et al.* 2014; Zhang *et al.* 2011) that have shown that high levels of cellular exposure to SWCNTs can alter sub-cellular components, including F-actin structures. Even though SWCNTs were present in *X. laevis* embryos at high local concentrations (up to ~ 2 mg mL⁻¹), there were no gross alterations to sub-cellular morphologies, including F-actin and ER. Instead, SWCNTs were located in peri-nuclear regions, as we have previously observed for these SWCNTs extracellularly exposed to and internalized into human and murine 2D cell culture (Boyer *et al.* 2013; Holt *et al.* 2011; Holt *et al.* 2012a; Holt *et al.* 2015; Holt *et al.* 2012b). Instead of SWCNTs aggregating into bundles which have been shown to be toxic (Kaiser *et al.* 2008; Liu *et al.* 2010) within cells due to strong van der Waals interaction energy between bare SWCNTs (Girifalco *et al.* 2000; Hough *et al.* 2004), they remained individually dispersed, which has been shown to play important roles in cellular and organism recovery from SWCNT exposure (Holt *et al.* 2012a; Holt *et al.* 2015; Jin *et al.* 2009; Kolosnjaj-Tabi *et al.* 2010; Liu *et al.* 2013; Ruggiero *et al.* 2010).

Extension to potential applications

Understanding how SWCNTs interact with developing embryos also yields insight into SWCNT interactions with stem cells, since injections occur at the 4-cell stage into pluripotent cells. Since embryos exposed to SWCNTs successfully developed throughout the length of the experiment, tailbud stage (stage 29), SWCNTs did not substantially alter pluripotent cell proliferation or differentiation.

These results indicate that purified, short, well dispersed SWCNTs have promise for many potential developmental and biotechnological applications. For example, the unique optical properties of SWCNTs exist in the NIR “biological window” (Hong *et al.* 2012; Welsher *et al.* 2011) which may allow for real-time imaging and/or photothermal strategies to probe development, or SWCNTs' high surface-area-to-volume ratio may allow for targeted, bioactive molecule delivery.

Conclusions

Injections of highly purified, length-selected, SWCNTs well dispersed with BSA into the animal region of *X. laevis* embryos were not toxic. SWCNTs were somewhat heterogeneously distributed throughout the animal cap ectoderm, specifically in the progeny of the cells that internalized the injection bolus. Confocal fluorescence imaging of cellular compartments co-registered to confocal Raman imaging of SWCNT G-band intensity revealed that SWCNTs were located in peri-nuclear regions, without grossly altering sub-cellular structures. SWCNTs remained well-dispersed in embryos, preserving properties of individualized SWCNTs and preventing deleterious effects of bundles. Our results suggest that purified, short, dispersed SWCNTs, after additional studies, may be candidate materials for diverse biological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by: the National Science Foundation (CMMI-1335417 [M.F.I.]; CAREER IOS-0845775 and CMMI-1100515 [L.A.D.]; CMMI-1300476 [K.N.D.]); and the National Institutes of Health (R01 HD044750 [L.A.D.]). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF or the NIH.

References

- Arnold MS, Green AA, Hulvat JF, Stupp SI, Hersam MC. Sorting carbon nanotubes by electronic structure using density differentiation. *Nat Nanotechnol.* 2006; 1:60–65. DOI: 10.1038/nnano.2006.52 [PubMed: 18654143]
- Bacchetta R, Santo N, Fascio U, Moschini E, Freddi S, Chirico G, Camatini M, Mantecca P. Nano-sized cuo, tio2 and zno affect xenopus laevis development. *Nanotoxicology.* 2012a; 6:381–398. DOI: 10.3109/17435390.2011.579634 [PubMed: 21574813]
- Bacchetta R, Tremolada P, Di Benedetto C, Santo N, Fascio U, Chirico G, Colombo A, Camatini M, Mantecca P. Does carbon nanopowder threaten amphibian development? *Carbon.* 2012b; 50:4607–4618. DOI: 10.1016/j.carbon.2012.05.047

- Bachilo SM, Strano MS, Kittrell C, Hauge RH, Smalley RE, Weisman RB. Structure-assigned optical spectra of single-walled carbon nanotubes. *Science*. 2002; 298:2361–2366. [PubMed: 12459549]
- Battigelli A, Ménard-Moyon C, Ros TD, Prato M, Bianco A. Endowing carbon nanotubes with biological and biomedical properties by chemical modifications. *Adv Drug Delivery Rev*. 2013; 65:1899–1920. DOI: 10.1016/j.addr.2013.07.006
- Becker ML, Fagan JA, Gallant ND, Bauer BJ, Bajpai V, Hobbie EK, Lacerda SH, Migler KB, Jakupciak JP. Length-dependent uptake of DNA-wrapped single-walled carbon nanotubes. *Adv Mater*. 2007; 19:939–945.
- Bilalis P, Katsigiannopoulos D, Avgeropoulos A, Sakellariou G. Non-covalent functionalization of carbon nanotubes with polymers. *RSC Adv*. 2014; 4:2911–2934. DOI: 10.1039/c3ra44906h
- Bourdiol F, Mouchet F, Perrault A, Fourquaux I, Datas L, Gancet C, Boutonnet JC, Pinelli E, Gauthier L, Flahaut E. Biocompatible polymer-assisted dispersion of multi walled carbon nanotubes in water, application to the investigation of their ecotoxicity using xenopus laevis amphibian larvae. *Carbon*. 2013; 54:175–191. DOI: 10.1016/j.carbon.2012.11.024
- Boyer PD, Holt BD, Islam MF, Dahl KN. Decoding membrane- versus receptor-mediated delivery of single-walled carbon nanotubes into macrophages using modifications of nanotube surface coatings and cell activity. *Soft Matter*. 2013; 9:758–764. DOI: 10.1039/C2SM26995C
- Bridges CM, Dwyer FJ, Hardesty DK, Whites DW. Comparative contaminant toxicity: Are amphibian larvae more sensitive than fish? *Bull Environ Contam Toxicol*. 2002; 69:562–569. DOI: 10.1007/s00128-002-0098-2 [PubMed: 12232728]
- Campagnolo L, Massimiani M, Magrini A, Camaioni A, Pietroiusti A. Physico-chemical properties mediating reproductive and developmental toxicity of engineered nanomaterials. *Curr Med Chem*. 2012; 19:4488–4494. [PubMed: 22998570]
- Campagnolo L, Massimiani M, Palmieri G, Bernardini R, Sacchetti C, Bergamaschi A, Vecchione L, Magrini A, Bottini M, Pietroiusti A. Biodistribution and toxicity of pegylated single wall carbon nanotubes in pregnant mice. *Part Fibre Toxicol*. 2013; 10doi: 10.1186/1743-8977-10-21
- Chen LQ, Hu PP, Zhang L, Huang SZ, Luo LF, Huang CZ. Toxicity of graphene oxide and multi-walled carbon nanotubes against human cells and zebrafish. *Sci China: Chem*. 2012; 55:2209–2216. DOI: 10.1007/s11426-012-4620-z
- Chen PH, Hsiao KM, Chou CC. Molecular characterization of toxicity mechanism of single-walled carbon nanotubes. *Biomaterials*. 2013; 34:5661–5669. DOI: 10.1016/j.biomaterials.2013.03.093 [PubMed: 23623425]
- Cheng JP, Chan CM, Veca LM, Poon WL, Chan PK, Qu LW, Sun YP, Cheng SH. Acute and long-term effects after single loading of functionalized multi-walled carbon nanotubes into zebrafish (danio rerio). *Toxicol Appl Pharmacol*. 2009; 235:216–225. DOI: 10.1016/j.taap.2008.12.006 [PubMed: 19133284]
- Cheng JP, Cheng SH. Influence of carbon nanotube length on toxicity to zebrafish embryos. *Int J Nanomed*. 2012; 7:3731–3739. DOI: 10.2147/ijn.s30459
- Cheng JP, Flahaut E, Cheng SH. Effect of carbon nanotubes on developing zebrafish (danio rerio) embryos. *Environ Toxicol Chem*. 2007; 26:708–716. DOI: 10.1897/06-272r.1 [PubMed: 17447555]
- Cheng JP, Gu YJ, Wang YJ, Cheng SH, Wong WT. Nanotherapeutics in angiogenesis: Synthesis and *in vivo* assessment of drug efficacy and biocompatibility in zebrafish embryos. *Int J Nanomed*. 2011; 6:2007–2021. DOI: 10.2147/IJN.S20145
- Curry S, Brick P, Franks NP. Fatty acid binding to human serum albumin: New insights from crystallographic studies. *Biochim Biophys Acta, Mol Cell Biol Lipids*. 1999; 1441:131–140. DOI: 10.1016/S1388-1981(99)00148-1
- Delgado IFD, Paumgartten FJR. Current challenges in toxicological research: Evaluation of the developmental toxicity of manufactured nanomaterials. *Vigilância Sanitária em Debate*. 2013; 1:11–24. DOI: 10.3395/vd.v1i4.114en
- Dresselhaus MS, Dresselhaus G, Saito R, Jorio A. Raman spectroscopy of carbon nanotubes. *Phys Rep*. 2005; 409:47–99. DOI: 10.1016/j.physrep.2004.10.006
- Dresselhaus, MS.; Dresselhaus, GE.; P, C. Science of fullerenes and carbon nanotubes. Academic Press; San Diego: 1996.

- Dubertret B, Skourides P, Norris DJ, Noireaux V, Brivanlou AH, Libchaber A. *In vivo* imaging of quantum dots encapsulated in phospholipid micelles. *Science*. 2002; 298:1759–1762. DOI: 10.1126/science.1077194 [PubMed: 12459582]
- Dulinska-Molak I, Mao HL, Kawazoe N, Chen GP. Variation of mechanical property of single-walled carbon nanotubes-treated cells explored by atomic force microscopy. *J Biomed Nanotechnol*. 2014; 10:651–659. DOI: 10.1166/jbn.2014.1745 [PubMed: 24734517]
- Dumont, J.; Schultz, TW.; Buchanan, M.; Kao, G. Short-term bioassays in the analysis of complex environmental mixtures III. In: Waters, MD.; Sanghu, SS.; Lewtas, J.; Claxton, L.; Chernoff, N.; Nesnow, S., editors. *Environmental science research*. Springer; New York: 1983. p. 393-405.
- Giannaccini M, Cuschieri A, Dente L, Raffa V. Non-mammalian vertebrate embryos as models in nanomedicine. *Nanomed -Nanotechnol*. 2014; 10:703–719. DOI: 10.1016/j.nano.2013.09.010
- Gimlich RL, Braun J. Improved fluorescent compounds for tracing cell lineage. *Dev Biol*. 1985; 109:509–514. DOI: 10.1016/0012-1606(85)90476-2 [PubMed: 2581834]
- Girifalco LA, Hodak M, Lee RS. Carbon nanotubes, buckyballs, ropes, and a universal graphitic potential. *Phys Rev B: Condens Matter Mater Phys*. 2000; 62:13104–13110. DOI: 10.1103/PhysRevB.62.13104
- Hamdoun A, Epel D. Embryo stability and vulnerability in an always changing world. *Proc Natl Acad Sci U S A*. 2007; 104:1745–1750. DOI: 10.1073/pnas.0610108104 [PubMed: 17264211]
- Heister E, Brunner EW, Dieckmann GR, Jurewicz I, Dalton AB. Are carbon nanotubes a natural solution? Applications in biology and medicine. *ACS Appl Mater Interfaces*. 2013; 5:1870–1891. DOI: 10.1021/am302902d [PubMed: 23427832]
- Heller DA, Baik S, Eurell TE, Strano MS. Single-walled carbon nanotube spectroscopy in live cells: Towards long-term labels and optical sensors. *Adv Mater*. 2005; 17:2793–2799. DOI: 10.1002/adma.200500477
- Heller DA, Barone PW, Swanson JP, Mayrhofer RM, Strano MS. Using raman spectroscopy to elucidate the aggregation state of single-walled carbon nanotubes. *J Phys Chem B*. 2004; 108:6905–6909. DOI: 10.1021/jp037690o
- Hersam MC. Progress towards monodisperse single-walled carbon nanotubes. *Nat Nanotechnol*. 2008; 3:387–394. DOI: 10.1038/nnano.2008.135 [PubMed: 18654561]
- Holt BD, Dahl KN, Islam MF. Quantification of uptake and localization of bovine serum albumin-stabilized single-wall carbon nanotubes in different human cell types. *Small*. 2011; 7:2348–2355. DOI: 10.1002/sml.201100437 [PubMed: 21626688]
- Holt BD, Dahl KN, Islam MF. Cells take up and recover from protein-stabilized single-wall carbon nanotubes with two distinct rates. *ACS Nano*. 2012a; 6:3481–3490. DOI: 10.1021/nn300504x [PubMed: 22458848]
- Holt BD, Dahl KN, Islam MF. Differential sub-cellular processing of single-wall carbon nanotubes via interfacial modifications. *J Mater Chem B*. 2015; 3:6274–6284. DOI: 10.1039/c5tb00705d
- Holt BD, McCorry MC, Boyer PD, Dahl KN, Islam MF. Not all protein-mediated single-wall carbon nanotube dispersions are equally bioactive. *Nanoscale*. 2012b; 4:7425–7434. DOI: 10.1039/C2NR31928D [PubMed: 23086474]
- Holt BD, Shams H, Horst TA, Basu S, Rape AD, Wang YL, Rohde GK, Mofrad MRK, Islam MF, Dahl KN. Altered cell mechanics from the inside: Dispersed single wall carbon nanotubes integrate with and restructure actin. *J Funct Biomater*. 2012c; 3:398–417. DOI: 10.3390/jfb3020398 [PubMed: 24955540]
- Holt BD, Short PA, Rape AD, Wang YL, Islam MF, Dahl KN. Carbon nanotubes reorganize actin structures in cells and *ex vivo*. *ACS Nano*. 2010; 4:4872–4878. DOI: 10.1021/nn101151x [PubMed: 20669976]
- Hong G, Lee JC, Robinson JT, Raaz U, Xie L, Huang NF, Cooke JP, Dai H. Multifunctional in vivo vascular imaging using near-infrared II fluorescence. *Nat Med (N Y, NY, U S)*. 2012; 18:1841–1846. DOI: 10.1038/nm.2995
- Hougaard KS, Jackson P, Kyjovska ZO, Birkedal RK, De Temmerman PJ, Brunelli A, Verleysen E, Madsen AM, Saber AT, Pojana G, Mast J, Marcomini A, Jensen KA, Wallin H, Szarek J, Mortensen A, Vogel U. Effects of lung exposure to carbon nanotubes on female fertility and

pregnancy. A study in mice. *Reprod Toxicol*. 2013; 41:86–97. DOI: 10.1016/j.reprotox.2013.05.006 [PubMed: 23714338]

Hough LA, Islam MF, Hammouda B, Yodh AG, Heiney PA. Structure of semidilute single-wall carbon nanotube suspensions and gels. *Nano Lett*. 2006; 6:313–317. DOI: 10.1021/nl051871f [PubMed: 16464056]

Hough LA, Islam MF, Janmey PA, Yodh AG. Viscoelasticity of single wall carbon nanotube suspensions. *Phys Rev Lett*. 2004; 93:168102.doi: 10.1103/PhysRevLett.93.168102 [PubMed: 15525036]

Huang XL, Zhang F, Sun XL, Choi KY, Niu G, Zhang GF, Guo JX, Lee S, Chen XY. The genotype-dependent influence of functionalized multiwalled carbon nanotubes on fetal development. *Biomaterials*. 2014; 35:856–865. DOI: 10.1016/j.biomaterials.2013.10.027 [PubMed: 24344357]

Hutchison, GR.; Ross, BL. Current *in vitro* models for nanomaterial testing. In: Tran, CL., editor. *Nanotoxicology: Progress toward nanomedicine*. second. Monteiro-Riviere NA: 2014. p. 357-376.

Islam MF, Milkie DE, Torrens ON, Yodh AG, Kikkawa JM. Magnetic heterogeneity and alignment of single wall carbon nanotubes. *Phys Rev B: Condens Matter Mater Phys*. 2005; 71:201401(R).doi: 10.1103/PhysRevB.71.201401

Islam MF, Rojas E, Bergey DM, Johnson AT, Yodh AG. High weight fraction surfactant solubilization of single-wall carbon nanotubes in water. *Nano Lett*. 2003; 3:269–273. DOI: 10.1021/nl025924u

Jackson P, Hougaard KS, Vogel U, Wu D, Casavant L, Williams A, Wade M, Yauk CL, Wallin H, Halappanavar S. Exposure of pregnant mice to carbon black by intratracheal instillation: Toxicogenomic effects in dams and offspring. *Mutat Res, Genet Toxicol Environ Mutagen*. 2012; 745:73–83. DOI: 10.1016/j.mrgentox.2011.09.018

Jackson P, Jacobsen NR, Baun A, Birkedal R, Kühnel D, Jensen KA, Vogel U, Wallin H. Bioaccumulation and ecotoxicity of carbon nanotubes. *Chem Cent J*. 2013; 7:154–154. DOI: 10.1186/1752-153x-7-154 [PubMed: 24034413]

Jariwala D, Sangwan VK, Lauhon LJ, Marks TJ, Hersam MC. Carbon nanomaterials for electronics, optoelectronics, photovoltaics, and sensing. *Chem Soc Rev*. 2013; 42:2824–2860. DOI: 10.1039/c2cs35335k [PubMed: 23124307]

Jin H, Heller DA, Sharma R, Strano MS. Size-dependent cellular uptake and expulsion of single-walled carbon nanotubes: Single particle tracking and a generic uptake model for nanoparticles. *ACS Nano*. 2009; 3:149–158. DOI: 10.1021/nn800532m [PubMed: 19206261]

Jin H, Heller DA, Strano MS. Single-particle tracking of endocytosis and exocytosis of single-walled carbon nanotubes in NIH-3T3 cells. *Nano Lett*. 2008; 8:1577–1585. DOI: 10.1021/nl072969s [PubMed: 18491944]

Johnston DE, Islam MF, Yodh AG, Johnson AT. Electronic devices based on purified carbon nanotubes grown by high-pressure decomposition of carbon monoxide. *Nat Mater*. 2005; 4:589–592. DOI: 10.1038/nmat1427 [PubMed: 16030521]

Kagan VE, Tyurina YY, Tyurin VA, Konduru NV, Potapovich AI, Osipov AN, Kisin ER, Schwegler-Berry D, Mercer R, Castranova V, Shvedova AA. Direct and indirect effects of single walled carbon nanotubes on raw 264.7 macrophages: Role of iron. *Toxicol Lett*. 2006; 165:88–100. DOI: 10.1016/j.toxlet.2006.02.001 [PubMed: 16527436]

Kaiser JP, Wick P, Manser P, Spohn P, Bruinink A. Single walled carbon nanotubes (SWCNT) affect cell physiology and cell architecture. *J Mater Sci : Mater Med*. 2008; 19:1523–1527. DOI: 10.1007/s10856-007-3296-y [PubMed: 17990080]

Kataura H, Kumazawa Y, Maniwa Y, Umezumi I, Suzuki S, Ohtsuka Y, Achiba Y. Optical properties of single-wall carbon nanotubes. *Synth Met*. 1999; 103:2555–2558. DOI: 10.1016/s0379-6779(98)00278-1

Kay, BK.; P, HB. *Xenopus laevis*: Practical uses in cell and molecular biology. Academic Press; New York: 1991.

Kim KT, Jang MH, Kim JY, Xing BS, Tanguay RL, Lee BG, Kim SD. Embryonic toxicity changes of organic nanomaterials in the presence of natural organic matter. *Sci Total Environ*. 2012; 426:423–429. DOI: 10.1016/j.scitotenv.2012.03.050 [PubMed: 22542237]

Kolosnjaj-Tabi J, Hartman KB, Boudjemaa S, Ananta JS, Morgant G, Szwarc H, Wilson LJ, Moussa F. *In vivo* behavior of large doses of ultrashort and full-length single-walled carbon nanotubes after

oral and intraperitoneal administration to swiss mice. *ACS Nano*. 2010; 4:1481–1492. DOI: 10.1021/nn901573w [PubMed: 20175510]

Leeuw TK, Reith RM, Simonette RA, Harden ME, Cherukuri P, Tsybolski DA, Beckingham KM, Weisman RB. Single-walled carbon nanotubes in the intact organism: Near-ir imaging and biocompatibility studies in drosophila. *Nano Lett*. 2007; 7:2650–2654. DOI: 10.1021/nl0710452 [PubMed: 17696559]

Liu BY, Campo EM, Bossing T. *Drosophila* embryos as model to assess cellular and developmental toxicity of multi-walled carbon nanotubes (MWCNT) in living organisms. *PLoS One*. 2014; 9:e88681(88681)–e88681(88689). DOI: 10.1371/journal.pone.0088681 [PubMed: 24558411]

Liu D, Yi C, Zhang D, Zhang J, Yang M. Inhibition of proliferation and differentiation of mesenchymal stem cells by carboxylated carbon nanotubes. *ACS Nano*. 2010; 4:2185–2195. DOI: 10.1021/nn901479w [PubMed: 20218664]

Liu J, Yu M, Zhou C, Zheng J. Renal clearable inorganic nanoparticles: A new frontier of bionanotechnology. *Mater Today*. 2013; 16:477–486. DOI: 10.1016/j.mattod.2013.11.003

Liu Z, Tabakman S, Welscher K, Dai H. Carbon nanotubes in biology and medicine: In vitro and in vivo detection, imaging and drug delivery. *Nano Res*. 2009; 2:85–120. DOI: 10.1007/s12274-009-9009-8 [PubMed: 20174481]

Marcon L, Riquet F, Vicogne D, Szunerits S, Bodart JF, Boukherroub R. Cellular and in vivo toxicity of functionalized nanodiamond in xenopus embryos. *J Mater Chem*. 2010; 20:8064–8069. DOI: 10.1039/c0jm01570a

Mouchet F, Landois P, Datsyuk V, Puech P, Pinelli E, Flahaut E, Gauthier L. International amphibian micronucleus standardized procedure (iso 21427-1) for in vivo evaluation of double-walled carbon nanotubes toxicity and genotoxicity in water. *Environ Toxicol*. 2011; 26:136–145. DOI: 10.1002/tox.20537 [PubMed: 20014232]

Mouchet F, Landois P, Puech P, Pinelli E, Flahaut E, Gauthier L. Carbon nanotube ecotoxicity in amphibians: Assessment of multiwalled carbon nanotubes and comparison with double-walled carbon nanotubes. *Nanomedicine*. 2010; 5:963–974. DOI: 10.2217/nnm.10.60 [PubMed: 20735230]

Mouchet F, Landois P, Sarremejean E, Bernard G, Puech P, Pinelli E, Flahaut E, Gauthier L. Characterisation and in vivo ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian xenopus laevis. *Aquat Toxicol*. 2008; 87:127–137. DOI: 10.1016/j.aquatox.2008.01.011 [PubMed: 18313771]

Murugesan S, Mousa SA, O'Connor LJ, Lincoln DW, Linhardt RJ. Carbon inhibits vascular endothelial growth factor- and fibroblast growth factor-promoted angiogenesis. *FEBS Lett*. 2007; 581:1157–1160. DOI: 10.1016/j.febslet.2007.02.022 [PubMed: 17331505]

Nieuwkoop, PD.; P, J. Normal tables of xenopus laevis (daudin). Elsevier North-Holland Biomedical Press; Amsterdam: 1967.

Nouara A, Wu QL, Li YX, Tang M, Wang HF, Zhao YL, Wang DY. Carboxylic acid functionalization prevents the translocation of multi-walled carbon nanotubes at predicted environmentally relevant concentrations into targeted organs of nematode caenorhabditis elegans. *Nanoscale*. 2013; 5:6088–6096. DOI: 10.1039/c3nr00847a [PubMed: 23722228]

O'Connell MJ, Bachilo SM, Huffman CB, Moore VC, Strano MS, Haroz EH, Rialon KL, Boul PJ, Noon WH, Kittrell C, Ma JP, Hauge RH, Weisman RB, Smalley RE. Band gap fluorescence from individual single-walled carbon nanotubes. *Science*. 2002; 297:593–596. DOI: 10.1126/science.1072631 [PubMed: 12142535]

Pietrojusti A, Massimiani M, Fenoglio I, Colonna M, Valentini F, Palleschi G, Camaioni A, Magrini A, Siracusa G, Bergamaschi A, Sgambato A, Campagnolo L. Low doses of pristine and oxidized single-wall carbon nanotubes affect mammalian embryonic development. *ACS Nano*. 2011; 5:4624–4633. DOI: 10.1021/nn200372g [PubMed: 21615177]

Pollard, TD.; Earnshaw, WC. Cell biology. Elsevier Inc; 2008.

Puech N, Blanc C, Grelet E, Zamora-Ledezma C, Maugey M, Zakri Cc, Anglaret E, Poulin P. Highly ordered carbon nanotube nematic liquid crystals. *J Phys Chem C*. 2011; 115:3272–3278. DOI: 10.1021/jp1102077

- Rodriguez-Fernandez L, Valiente R, Gonzalez J, Villegas JC, Fanarraga ML. Multiwalled carbon nanotubes display microtubule biomimetic properties in vivo, enhancing microtubule assembly and stabilization. *ACS Nano*. 2012; 6:6614–6625. DOI: 10.1021/nn302222m [PubMed: 22769231]
- Roman D, Yasmeen A, Mireuta M, Stiharu I, Al Moustafa AE. Significant toxic role for single-walled carbon nanotubes during normal embryogenesis. *Nanomed -Nanotechnol*. 2013; 9:945–950. DOI: 10.1016/j.nano.2013.03.010
- Ruggiero A, Villa CH, Bander E, Rey DA, Bergkvist M, Batt CA, Manova-Todorova K, Deen WM, Scheinberg DA, McDevitt MR. Paradoxical glomerular filtration of carbon nanotubes. *Proc Natl Acad Sci U S A*. 2010; 107:12369–12374. DOI: 10.1073/pnas.0913667107 [PubMed: 20566862]
- Sargent LM, Hubbs AF, Young SH, Kashon ML, Dinu CZ, Salisbury JL, Benkovic SA, Lowry DT, Murray AR, Kisin ER, Siegrist KJ, Battelli L, Mastovich J, Sturgeon JL, Bunker KL, Shvedova AA, Reynolds SH. Single-walled carbon nanotube-induced mitotic disruption. *Mutat Res, Genet Toxicol Environ Mutagen*. 2012; 745:28–37. DOI: 10.1016/j.mrgentox.2011.11.017
- Saria R, Mouchet F, Perrault A, Flahaut E, Laplanche C, Boutonnet JC, Pinelli E, Gauthier L. Short term exposure to multi-walled carbon nanotubes induce oxidative stress and DNA damage in xenopus laevis tadpoles. *Ecotoxicol Environ Saf*. 2014; 107:22–29. DOI: 10.1016/j.ecoenv.2014.05.010 [PubMed: 24905693]
- Shams H, Holt BD, Mahboobi SH, Jahed Z, Islam MF, Dahl KN, Mofrad MRK. Actin reorganization through dynamic interactions with single-wall carbon nanotubes. *ACS Nano*. 2014; 8:188–197. DOI: 10.1021/nn402865e [PubMed: 24351114]
- Sive, HL.; Grainger, RM.; Harland, RM. Early development of xenopus laevis: A laboratory manual. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2000.
- Vankayala R, Kalluru P, Tsai HH, Chiang CS, Hwang KC. Effects of surface functionality of carbon nanomaterials on short-term cytotoxicity and embryonic development in zebrafish. *J Mater Chem B*. 2014; 2:1038–1047. DOI: 10.1039/c3tb21497d
- Villegas JC, Álvarez-Montes L, Rodríguez-Fernández L, González J, Valiente R, Fanarraga ML. Multiwalled carbon nanotubes hinder microglia function interfering with cell migration and phagocytosis. *Adv Healthcare Mater*. 2014; 3:424–432. DOI: 10.1002/adhm.201300178
- Welsher K, Sherlock SP, Dai H. Deep-tissue anatomical imaging of mice using carbon nanotube fluorophores in the second near-infrared window. *Proc Natl Acad Sci U S A*. 2011; 108:8943–8948. DOI: 10.1073/pnas.1014501108 [PubMed: 21576494]
- Wierzbicki M, Sawosz E, Grodzik M, Prasek M, Jaworski S, Chwalibog A. Comparison of anti-angiogenic properties of pristine carbon nanoparticles. *Nanoscale research letters*. 2013; 8:195.doi: 10.1186/1556-276x-8-195 [PubMed: 23618362]
- Yaron P, Holt B, Short P, Losche M, Islam M, Dahl K. Single wall carbon nanotubes enter cells by endocytosis and not membrane penetration. *J Nanobiotechnol*. 2011; 9:45(01)–45(15). DOI: 10.1186/1477-3155-9-45
- Zhang Y, Wang B, Meng X, Sun G, Gao C. Influences of acid-treated multiwalled carbon nanotubes on fibroblasts: Proliferation, adhesion, migration, and wound healing. *Ann Biomed Eng*. 2011; 39:414–426. DOI: 10.1007/s10439-010-0151-y [PubMed: 20824344]

Figure 1.

Raman imaging of the localization of BSA-dispersed SWCNTs in explanted animal cap ectoderm tissues. (A) Zoomed out views of animal cap ectoderm reveal that SWCNTs are distributed throughout the explants, with some regions having high concentrations. Scale bars are 100 μm . (B) Images at increasing zoom and spatial resolution show detailed localization of SWCNTs. Scale bars are 10 μm . (C) Raman spectra from the positions indicated demonstrating the RBMs (200 – 250 cm^{-1}), D-band ($\sim 1300 \text{ cm}^{-1}$), G-band ($\sim 1591 \text{ cm}^{-1}$), G'-band ($\sim 2600 \text{ cm}^{-1}$) and NIR fluorescence (2100 – 3000 cm^{-1}), in addition to numerous other peaks arising from molecules of the animal cap ectoderm. The presence of RBMs $< 250 \text{ cm}^{-1}$ and fluorescence confirm that SWCNTs remained individually dispersed.

Figure 2.

3D spatial localization of SWCNTs within animal cap ectoderm. Images are maximum projections in Z-direction, Y-direction (bottom), and X-direction (right). Note that all Y- and X-direction projections are anisotropically stretched by 5× as indicated to increase visual clarity. Confocal fluorescence Z-stacks of F-actin were co-registered to confocal Z-stacks of the Raman G-band intensity of SWCNTs. Z-stacks of phase contrast images were also acquired and are included in a separate overlay for clarity. The cyan dashed boxes indicate the spatial regions in which Raman spectra were acquired. Note that SWCNT signal is not co-registered with altered F-actin structures or highly phase-dense regions.

Figure 3.

Cellular distribution of SWCNTs. Fluorescently labeled dextrans, which are known to localize within the progeny of cells that internalized the injection volume, were also microinjected with SWCNTs. Animal caps were dissociated to allow for individual cell imaging and unambiguous determination of co-localization. Confocal fluorescence images of F-actin (present in all cells) and dextrans (present in the progeny of cells that internalized the injection volume) were co-registered to confocal Raman images of SWCNT G-band and RBM intensities. The co-localization of SWCNTs and dextrans confirms that SWCNTs are present in cells that internalize the bolus but not in neighboring cells. Intense RBM signal $< 250 \text{ cm}^{-1}$ demonstrates that SWCNTs remain individualized (*i.e.*, do not bundle). SNR is an abbreviation for signal-to-noise ratio. A representative extended scan shows the peaks characteristic of SWCNTs and also SWCNT NIR fluorescence ($\sim 2100\text{--}3000 \text{ cm}^{-1}$).

Figure 4. SWCNT sub-cellular localization. Fluorescence images of dissociated animal cap ectoderm tissues co-registered to SWCNT Raman G-band intensity images demonstrate that SWCNTs are located in peri-nuclear regions. Zoomed-in, high spatial resolution Raman images further demonstrate that SWCNTs do not alter F-actin or ER structures.

Table 1

Toxicity of BSA and BSA-dispersed SWCNTs in *X. laevis* embryos.

Group	8-cell	St. 11	St. 14	St. 29
Control	20/20 healthy	20/20 healthy	20/20 healthy	20/20 healthy
4 nL	20/20 healthy	1/20 stalled gastrulation	20/20 healthy	20/20 healthy
1.0 wt.% BSA	8 nL	20/20 healthy	6/20 stalled gastrulation	2/20 exogastrulae* 14/18 shortened axes
	16 nL	20/20 healthy	12/20 stalled gastrulation	15/20 exogastrulae* 5/5 shortened axes
	SWCNTs-BSA 4 nL of 700 µg mL ⁻¹	20/20 healthy	20/20 healthy	20/20 healthy

* Exogastrulae are embryos that fail to gastrulate, specifically in which the endomesoderm and yolk plug are extruded outwards. These embryos were removed from culture upon observation.