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Expression patterns of *Hox10* paralogous genes during lumbar spinal cord development

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Abstract:
We have examined the expression of three paralogous Hox genes from E11.5 through E15.5 in the mouse spinal cord. These ages coincide with major phases of spinal cord neurogenesis, neuronal differentiation, cell migration, gliogenesis, and motor neuron cell death. The three genes, Hoxa10, Hoxc10, and Hoxd10, are all expressed in the lumbar spinal cord and have distinct expression patterns. Mutations in these three genes are known to affect motor neuron patterning. All three genes show lower levels of expression at the rostral limits of their domains, with selective regions of higher expression more caudally. Hoxa10 and Hoxd10 expression appears confined to postmitotic cell populations in the intermediate and ventral gray, while Hoxc10 is also expressed in proliferating cells in the dorsal ventricular zone. Hoxc10 and Hoxd10 expression is clearly excluded from the lateral motor columns at rostral lumbar levels but is present in this region more caudally. Double labeling demonstrates that Hoxc10 expression is correlated with ventrolateral LIM gene expression in the caudal part of the lumbar spinal cord.
The mammalian Hox gene family consists of 39 genes organized in four linkage groups on four separate chromosomes (Scott, 1992; reviewed in Capecchi, 1997.). Paralogous Hox genes occupy the same relative position within each linkage group and show a high degree of sequence similarity (reviewed in Bürglin, 1994). Hox genes encode transcription factors and are expressed along the anteroposterior axis in broad domains encompassing both neural tube and axial mesoderm (Duboule and Dollé, 1989; reviewed in Izpisúa-Belmonte and Duboule, 1992; Schilling and Knight, 2001; Carpenter, 2002). The global position of Hox gene expression domains are likely established in response to retinoic acid or fibroblast growth factor signaling (reviewed in Deschamps et al., 1999) while the specific expression of Hox genes within the neural tube may be initiated by signaling from adjacent mesoderm or from the node (Ensini et al., 1998; Liu et al., 2001; Omelchenko and Lance-Jones, 2003). At early stages of development, individual Hox genes are expressed over significant lengths of the AP extent of the neural tube, while at later stages these domains become more limited (e.g. Murphy et al., 1989; Peterson et al., 1994; Tiret et al., 1998; Liu et al., 2001). In addition to sequence similarities, paralogous genes also show overlapping domains of expression in the neural tube (reviewed in Carpenter, 2002).

Inactivating Hox genes produces a wide array of phenotypes in the hindbrain and spinal cord suggesting complex roles for these genes in governing nervous system development. Knockout phenotypes include segmental deletions, motor neuron respecification, and projection errors (e.g. Carpenter et al., 1993; Goddard et al., 1996; Tiret et al., 1998; Wahba et al., 2000; Lin and Carpenter, 2003). One interesting observation is that gene knockouts typically affect subsets of neurons and/or glial cells within nervous system regions or segments, suggesting that Hox gene function may be required only within specific cell populations. These phenotypes suggest an intricate involvement of Hox genes in defining segmental and cellular identity in the developing nervous system.

Beyond identification of broad domains of early expression, little attention has been paid to the regional pattern or dynamics of Hox gene expression, making it difficult to relate these patterns to the knockout phenotypes observed in the nervous system. The onset of Hox gene expression in the nervous system is typically quite early, with most genes expressed on or slightly before embryonic day (E) 8 in the mouse; the expression
of many Hox genes persists into late embryogenesis as we report here. In this study, we have examined the expression of all three members of the paralogous Hox10 gene family, Hoxa10, Hoxc10, and Hoxd10, though multiple stages in spinal cord development. These genes were of particular interest first, because they constitute a complete paralogous family, and second, because knockout mouse lines exist for all three genes (Carpenter et al., 1997; Wahba et al., 2000; Hostikka and Carpenter, in preparation), allowing analysis of the specific function of these genes in nervous system development. All three genes are expressed at lumbar levels of the spinal cord; inactivation of these genes alone or in combination with each other alters at least one specific population of cells, the motor neurons (Carpenter et al., 1997; Wahba et al., 1999; Lin and Carpenter, 2003). In the current study, we demonstrate that despite a largely shared anteroposterior domain of expression, each of these Hox genes has a unique temporal and dorsoventral pattern of expression. In addition, we demonstrate that Hox gene expression is not uniform throughout its entire anteroposterior domain. These expression patterns support a role for Hox10 genes in establishing lumbar spinal cord patterning and suggest that regional differences in expression may underlie the different phenotypes observed following mutation of these paralogous genes. In addition, our findings show that only some subsets of motor neurons within a global expression domain express Hox10 genes, supporting studies of knockout mice that demonstrate that effects on specific populations of these cells.

1. Results and discussion:

In situ hybridization was used to examine the expression of three paralogous genes, Hoxa10, Hoxc10, and Hoxd10 in the developing lumbar spinal cord during mid-to-late mouse embryogenesis. Prior studies in mouse embryos (Bensen et al., 1995; Dollé and Duboule, 1989; Peterson et al., 1992; Hostikka and Capecchi, 1998; reviewed in Carpenter, 2002) have shown that these genes are expressed as early as E8.5; our studies were confined to later stages of development during active neurogenesis, neural differentiation, cell migration, and gliogenesis in the lumbar spinal cord. At E11.5, the initial timepoint examined in our studies, ventral neurogenesis, including production of the motor neurons is largely complete in the ventral spinal cord (Lance-Jones, 1982) but dorsal neurogenesis is still active (Nornes and Carry, 1978). At this age, Hoxa10 is
expressed along the dorsal margin of the developing spinal cord in the developing dorsal horn and in a small ventromedial domain lateral to the floorplate (Figure 1). Both of these areas of expression appear throughout the anteroposterior extent of the lumbar spinal cord. In contrast, \textit{Hoxc10} and \textit{Hoxd10} appear largely restricted to the ventrolateral spinal cord in the developing ventral horn; this region corresponds to the location of the lateral motor columns. \textit{Hoxd10} expression appears to expand slightly more dorsally than \textit{Hoxc10}, suggesting this gene might also be expressed in ventral interneurons. \textit{Hoxa10}, \textit{Hoxc10}, and \textit{Hoxd10} are not expressed in the ventricular zone at this age. This is interesting because \textit{Hox} genes have been proposed as regulators of cell proliferation in developing limb mesodermal tissue (e.g. Kmita et al., 2005; Fromental-Ramain et al., 1996); exclusion of \textit{Hox10} gene expression from this region suggests that, at E11.5, \textit{Hox10} genes are not expressed in proliferating neural progenitor populations. At this age, few differences are noted in expression patterns throughout the anteroposterior extend of the expression domains.

By E12.5, the expression of all three \textit{Hox10} genes has increased and both gradients of expression along the rostrocaudal axis and distinct regional differences along the anteroposterior axis can be detected. At this stage of lumbar spinal cord development, the somatic motor neurons have essentially all been produced, but active neurogenesis continues in the dorsal horn. Active gliogenesis also is reported at this age, with the first appearance of PDGFRα+ oligodendrocyte precursors in the ventral neuroepithelium (reviewed in Woodruff et al., 2001). Prior studies in sagittal sections demonstrated anterior limits of expression for these three \textit{Hox} genes at the thoracic/lumbar boundary (Duboule and Dolly, 1989; Benson et al., 1995; Peterson et al., 1992; Hostikka and Capecchi, 1998); our current observations suggest minimal to no expression of \textit{Hoxa10}, \textit{Hoxc10}, or \textit{Hoxd10} at thoracic levels (Figure 2). In the L1 segment at the rostral end of the lumbar spinal cord, \textit{Hoxa10} is expressed at relatively low levels in a heterogeneous distribution. \textit{Hoxa10} expression is present in the posterior extent of the intermediolateral cell column (IML), in ventral interneurons, and adjacent to the dorsal ventricular zone. (Figure 2B). Dorsal expression corresponds to the position of the medial extent of dI1-2 dorsal interneurons (Helms and Johnson, 2003). Expression increases more caudally in L2 and L4, with a ventromedial group of cells lateral to the ventricular zone and dorsal to the floorplate exhibiting the highest levels of expression.
This region abuts the edge of the floorplate, suggesting these may be $V_3$ interneurons (Jessell, 2000). In L2 and L4, Hoxa10 dorsal expression widens into a broad band compared to expression in L1 and overlaps the lateral margins of the ventricular zone. At the L4 lumbar segmental level, Hoxa10 expression appears to be excluded from the ventrolateral spinal cord (Figure 2D, asterisks), from the region occupied by gluteal and hamstring motor neurons (McHanwell and Biscoe, 1981).

Hoxc10 appears more widely expressed than Hoxa10 at the L1 level at E12.5 (Figure 2F-H). At this level, Hoxc10 expression is present in the caudal extent of the IML, as well as in more medial dorsal and ventral interneurons. Hoxc10 is also expressed in the medial part of the lateral motor column (LMC) and in the medial motor column (MMC; Figure 2G, arrowheads). Dorsally, Hoxc10 is expressed lateral to the ventricular zone, overlapping Hoxa10 expression. In the L2 segment, Hoxc10 expression is widely expressed in postmitotic cells including $V_0$ and $V_1$ interneurons, but is excluded from lateral LMC (Figure 2G, asterisks). Dorsally, expression is evident at high levels lateral to the ventricular zone and at low levels in the ventricular zone itself. This contrasts to the exclusion of Hoxa10 and Hoxd10 expression from the ventricular zone. More caudally, at L4, Hoxc10 expression is evident in most ventral neurons including LMC neurons (Figure 2H, arrowhead). This pattern suggests the specific exclusion of Hoxc10 expression from lumbar motor neurons at rostral levels, corresponding to the position of the quadriceps femoris motor pools (McHanwell and Biscoe, 1981). Similar heterogeneity of expression has not previously been described for Hoxc10 (Hostikka and Capecchi, 1998; Liu et al., 2001).

Hoxd10 expression appears largely confined to postmitotic cells in the intermediate and ventral spinal cord. This contrasts with the Hoxd10 expression pattern reported for chick embryos, where most postmitotic cells appear to express Hoxd10 (Lance-Jones et al., 2001). Levels of expression are relatively low in L1, but increase in more posterior segments. At the L2 segmental level, Hoxd10 expression appears excluded from the LMC (Figure 2K, asterisk), but is present at high levels across the remainder of the ventral spinal cord. As with Hoxc10, Hoxd10 expression also appears excluded from the quadriceps femoris motor pools, but is present in more caudal motor pools.
At E13.5, *Hox10* gene expression persists with further refinement of the expression patterns seen at earlier stages of development. At this stage, neurogenesis is largely complete, but cell migration and gliogenesis continue. In rostral lumbar spinal cord, *Hoxa10* expression is apparent in the posterior intermediolateral cell column and at the edge of the ventral ventricular zone, overlapping the position of *V₃* interneurons (Figure 3A, arrowhead). Dorsally, *Hoxa10* is expressed at lower levels adjacent to the ventricular zone. More caudally, in the L2 and L4 segments, *Hoxa10* expression is maintained at high levels only in *V₃* interneurons, while lower levels of expression are apparent in more dorsal interneurons (Figure 3B, C). *Hoxc10* is more widely expressed than *Hoxa10* at E13.5, with expression apparent in the majority of ventral interneurons at all lumbar levels (Figure 3D-F). Dorsal *Hoxc10* expression is apparent in a thin band of medial interneurons and at low levels in the dorsal ventricular zone. As seen at E12.5, *Hoxc10* is not expressed in the quadriceps femoris motor pools of the rostral LMC, but is expressed in the LMC at more caudal levels (Figure 3E, F). In contrast to both *Hoxa10* and *Hoxc10*, *Hoxd10* expression is confined to the ventral spinal cord at E13.5 (Figure 3G-I). At midlumbar levels, a *Hoxd10* expression is clearly excluded from the LMC, while surrounding intermediate and ventromedial regions of the spinal cord show high levels of *Hoxd10* expression (Figure 3H). Again, the region of exclusion corresponds to the position of the quadriceps femoris motor pools. At more caudal levels, *Hoxd10* expression is apparent across the mediolateral extent of the intermediate and ventral spinal cord, although levels of expression appear somewhat lower laterally (Figure 3I).

The segregation of *Hoxd10* expression to specific motor pools is particularly interesting in light of peroneal nerve phenotypes observed in *Hoxd10* mutants (Carpenter et al., 1997). In 30% of *Hoxd10* mutants, the peroneal nerve is absent. Motor neurons giving rise to this nerve are positioned caudally in the lumbar spinal cord, corresponding to the levels in which *Hoxd10* is expressed in motor neurons. Therefore, inactivation of *Hoxd10* specifically affects motor neurons that express the gene.

E15.5 corresponds with a period of active gliogenesis and the end of the major period of motor neuron cell death in the spinal cord (Lance-Jones, 1982; Woodruff et al., 2001). By this time, all *Hox10* gene expression is confined largely to the ventral part of the spinal cord. At the L1 level, *Hoxa10* shows a somewhat patchy distribution, while *Hoxc10* and *Hoxd10* appear more uniformly expressed. *Hoxc10* expression extends
farther dorsally than either *Hoxa10* or *Hoxd10*. *Hoxd10* expression is restricted to intermediate and ventral spinal cord, with roughly uniform levels of expression along the anteroposterior extent of the lumbar spinal cord. These observations implicate a continued requirement for *Hox10* gene expression in more ventral parts of the spinal cord.

*Hoxc10* expression was also examined using β-galactosidase expression from a *lacZ* reporter gene targeted to the *Hoxc10* locus (Hostikka et al., manuscript in preparation). Mice heterozygous for the *lacZ* insertion do not have overt behavioral or anatomical phenotypes and β-galactosidase expression largely mirrors *Hoxc10* expression detected using *in situ* hybridization (Figure 5). β-galactosidase expression is evident from E11.5 – E15.5. At E11.5, β-galactosidase expression is seen at midlumbar levels in the ventrolateral spinal cord (Figure 5B) similar to mRNA expression detected using *in situ* hybridization (Figure 1D). One difference is the presence of β-galactosidase expression along the lateral edge of the spinal cord (Figure 5A, B). β-galactosidase expression is also evident in the dorsal ventricular zone at E11.5, albeit at low levels. At E12.5-E15.5, β-galactosidase expression also largely mirrors *Hoxc10* mRNA expression. β-galactosidase expression is absent from the region of the developing lateral motor columns at the L2 level, while it is present laterally at more caudal levels, similar to *Hoxc10* mRNA expression patterns. β-galactosidase expression along the lateral edge of the spinal cord is present at relative high levels at E12.5 but decreases significantly at E13.5. Therefore, while β-galactosidase expression largely mirrors *Hoxc10* mRNA expression, a few differences are evident. These may reflect more stable β-galactosidase expression as compared to mRNA expression or may suggest some alteration in *Hoxc10* gene expression induced by the *lacZ* gene insertion. Further studies are currently in progress to explore these possibilities (Hostikka et al., manuscript in preparation).

To determine if *Hoxc10* expression (or lack thereof) correlated with the presence of motor neurons, double labeling studies were performed combining histochemical detection of β-galactosidase and immunohistochemical detection of Islet-1 and Islet-2, LIM homeodomain proteins that are expressed early in motor neuron development (Tsuchida et al., 1994). At midlumbar levels, Islet-1/2 expression and *Hoxc10*-driven β-galactosidase expression were segregated (Figure 5G, G’), suggesting that *Hoxc10* is not expressed in LMC motor pools at this spinal cord level. However, at more caudal levels,
Islet-1/2 was coexpressed with β-galactosidase, suggesting that some motor pools do express *Hoxc10* (Figure 5G, H). Observations at higher magnification suggest that within these regions of overlap at least some Islet-1/2-positive cells also express *Hoxc10* (Figure 5H, inset).

In summary, our results demonstrate that patterns of *Hox10* gene expression are dynamic in both time and space in the embryonic mouse lumbar spinal cord. While *Hoxc10* and *Hoxd10* expression have been examined at gross levels in mouse spinal cord (Peterson et al., 1992; Dollé and Duboule, 1989) and more extensively in chick (Liu et al., 2001; Lance-Jones et al., 2001; Omelchenko and Lance-Jones, 2003), prior studies have not demonstrated the regional selectivity we document here regarding gene expression in specific motor pools. These variations may reflect species differences between mouse and chick. Studies examining *Hoxc8* expression in mouse spinal cord (Tiret et al., 1998) have also demonstrated regional expression of *Hox* genes in specific motor pools. These observations, coupled with the findings that only subsets of motor pools are affected in *Hox* knockout mice, support the hypothesis that that *Hox* gene activity may be required for specification or identity of distinct motor pools.

2. Experimental procedures:

2.1 In situ hybridization

*Hoxa10, Hoxc10, and Hoxd10* probe templates were generated by subcloning PCR-amplified inserts into pBluescript (Stratagene, *Hoxd10*) or T-Easy (Promega, *Hoxa10* and *Hoxc10*). Primer pairs were designed to amplify sequences from the first exon of each gene to avoid overlap with the highly conserved homeodomain sequences encoded by exon 2. Primer pairs were as follows: *Hoxa10* forward - 5’-TGC GCA GAA CAT CAA AGA AG -3’, *Hoxa10* reverse - 5’- CGG CGA AGC TTT ACT GTT TT -3’, *Hoxc10* forward - 5’-GAG CGC TAT AAC CGT AAC GC -3’, *Hoxc10* reverse - 5’-CTG AGG CGA TTC C AG ATG TT -3’, *Hoxd10* forward - 5’- TTC CAT GCC ACC ACC TAG CGA TTC CAG ATG TT-3’, *Hoxd10* reverse - 5’-TTC CAT GCC ACC ACC TAG CGC AG-3’, *Hoxd10* reverse – 5’-TTC GGG CTC CTG GGC GCT CGC -3’. Templates were linearized by restriction digest and antisense RNA probes transcribed with T7 or SP6 polymerase in the presence of digoxigenin-labeled dUTP (Roche).
C57Bl/6 mouse embryos collected from timed pregnancies were fixed for 4 hours in 4% paraformaldehyde (PFA)/1x PBS, washed, and infiltrated with 30% sucrose. Embryos were embedded in OCT (Tissue Tek), frozen and sectioned at 20 µm. Cryosections were collected directly to Superfrost Plus glass slides (Fisher Scientific), postfixed with 4% PFA/1x PBS, washed and hybridized with labeled RNA probes at 72°C for 15-18 hours. Digoxigenin was detected using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and visualized by reacting with NBT/BCIP (Roche). A minimum of three embryos were examined at each embryonic age using each of the three probes.

2.2 ß-galactosidase expression and immunolabeling
Mouse embryos heterozygous for a lacZ insertion into the Hoxc10 gene were collected from intercrosses of heterozygous parents or from crosses of wild-type/mutant parents. The Hoxc10 lacZ insertion has been maintained on a C57Bl/6 background for more than 6 generations. Embryos were fixed for 20 minutes in 4% PFA/1x PBS, washed and infiltrated with 30% sucrose, frozen in OCT and sectioned at 20 µm. Sections were reacted overnight at 37°C using 1 mg/ml X-gal in 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆·3H₂O, 1 mM MgCl₂, 0.01% DOC, and 0.02% Igepal (Sigma). Slides were then washed briefly and immunolabeled for Islet-1 and Islet-2 expression using the 40.2D6 antibody (Developmental Studies Hybridoma Bank), which recognizes both proteins. Islet-1/2 labeling was detected using HRP-conjugated goat-anti-mouse antibodies (Jackson Immunoresearch) and visualized by reacting with 0.5 mg/ml diaminobenzidine and 0.03% hydrogen peroxide.

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References


**Figure Legends**

*Figure 1:* **Hoxa10**, **Hoxc10**, and **Hoxd10** expression in E11.5 lumbar spinal cord. Gene expression was detected using *in situ* hybridization. In this and subsequent figures, spinal segmental level is indicated at the top of the figure. **Hoxa10** (A, B) is expressed in the superficial dorsal horn (double arrows) and in a ventromedial patch (arrowheads). **Hoxc10** (C, D) and **Hoxd10** (E, F) are both expressed ventrolaterally (arrows), overlapping the position of the lateral motor columns. Scale bar, 150 µm.

*Figure 2:* **Hoxa10**, **Hoxc10**, and **Hoxd10** expression in E12.5 spinal cord. None of the genes are expressed at thoracic spinal cord levels (T12 – A, E, I). All three genes are expressed at lower levels in the rostral lumbar cord (L1 – B, F, J) with increasing levels of expression more caudally (L2 and L4, C, D, G, H, K, L). **Hoxa10** (A-D) is present overlapping the caudal extend of the IML (B, single arrow), dorsally lateral to the ventricular zone (B, C, D, double arrows) and ventrally adjacent to the floorplate (B, C, D, arrowhead). **Hoxa10** expression is excluded from the LMC at caudal levels (D, asterisk). **Hoxc10** (E-H) is expressed at low levels in the dorsal ventricular zone (F, G, H, single arrow), at higher levels lateral to the ventricular zone (F, G, H, double arrows), and throughout the intermediate and ventral gray. **Hoxc10** is not expressed in the LMC in the rostral lumbar spinal cord (G, asterisks), but is expressed in the MMC (G, arrowheads) and in the caudal LMC (H, arrowhead). **Hoxd10** (H-J) is expressed throughout the intermediate and ventral gray, but is excluded from the rostral LMC (K, asterisk). Section in K is slightly oblique, therefore **Hoxd10** expression is apparent in the LMC on the opposite side (K, arrowhead). Scale bar, 150 µm.

*Figure 3:* **Hoxa10**, **Hoxc10** and **Hoxd10** expression in E13.5 lumbar spinal cord. **Hoxa10**, **Hoxc10**, and **Hoxd10** are expressed at lower levels rostrally (L1 – A, D, E) and at higher levels caudally (L2 and L4 – B, C, E, F, H, I) in the lumbar spinal cord. In the L1 segment, **Hoxa10** is expressed at high levels in the caudal IML (A, arrow), ventromedially adjacent to the floorplate (A, arrowhead), and at lower levels throughout dorsomedial, intermediate, and ventral gray. **Hoxa10** and **Hoxc10** are both expressed dorsally lateral to the ventricular zone (B, E double arrows). **Hoxc10** and **Hoxd10** are
excluded from the ventrolateral spinal cord at midlumbar levels (E, H, asterisks), but are expressed in this region in more caudal sections (F, I, arrowheads). Scale bar, 150 µm.

Figure 4: **Hoxa10** (A, B), **Hoxc10** (C, D) and **Hoxd10** (E, F) expression in E15.5 lumbar spinal cord. All three genes are all expressed ventrally in the lumbar spinal cord. **Hoxa10** is also expressed at low levels in the dorsal horn (B, asterisk), and **Hoxc10** is present in a small dorsomedial region (C, arrow). Scale bar, 150 µm

Figure 5: **Hoxc10**-driven *lacZ* expression in the lumbar spinal cord. At E11.5 (A, B) *lacZ* expression is present in the L2 and L4 spinal segments in the ventrolateral spinal cord (arrowheads), at the lateral edge of the spinal cord (dashed arrows) and in the dorsal ventricular zone (solid arrows). At E12.5 (C, D), a dorsal band of expression (double arrows) flanks the ventricular zone (arrows). **Hoxc10** expression is excluded from the ventrolateral spinal cord in the L2 spinal segment (C, asterisks) but is present in this region at the L4 level (D, arrowhead). At E13.5 (E, F) ventricular zone expression is prominent (arrow); expression continues to be excluded ventrolaterally at the L2 level (E, asterisks) but is present more caudally (F, arrowhead). Double labeling with anti-Islet-1/2 antibodies (G, G’, H) demonstrates segregation of β-galactosidase and Islet-1/2 expression in the LMC motor pools. A longitudinal section (G) illustrates little overlap between Islet-1/2 expression (brown) and **Hoxc10**-driven β-galactosidase expression (blue) at more rostral levels (left side), but substantial overlap caudally (right side). Arrows in G indicate the level of sections in G’ and H. At the L2 level (G’), little overlap is seen between Islet-1/2 and **Hoxc10**-driven β-galactosidase (ovals), while at the L4 level (H), **Hoxc10**-driven β-galactosidase and Islet-1/2 expression overlap within the LMC (ovals). Higher magnification (inset in H) illustrates colocalization of Islet-1/2 and β-galactosidase in some cells (arrowheads). At E15.5, *lacZ* expression is present throughout the intermediate and ventral gray and along the dorsal midline (I, J, arrows). Scale bars indicate 150 µm in A-F, 50 µm in G, H, and 100 µm in I, J.