C4'/H4' selective, non-uniformly sampled 4D HC(P)CH experiment for sequential assignments of 13C-labeled RNAs

Saurabh Saxena · Jan Stanek · Mirko Cevec · Janez Plavec · Wiktor Kożmiński

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Abstract A through bond, C4'/H4' selective, “out and stay” type 4D HC(P)CH experiment is introduced which provides sequential connectivity via H4'(_i)–C4'(_i)–C4'(_i−1)–H4'(_i−1) correlations. The 31P dimension (used in the conventional 3D HCP experiment) is replaced with evolution of better dispersed C4' dimension. The experiment fully utilizes 13C-labeling of RNA by inclusion of two C4' evolution periods. An additional evolution of H4' is included to further enhance peak resolution. Band selective 13C inversion pulses are used to achieve selectivity and prevent signal dephasing due to the of C4'–C3' and C4'–C5' homonuclear couplings. For reasonable resolution, non-uniform sampling is employed in all indirect dimensions. To reduce sensitivity losses, multiple quantum coherences are preserved during shared-time evolution and coherence transfer delays. In the experiment the intranucleotide peaks are suppressed whereas inter-nucleotide peaks are enhanced to reduce the ambiguities. The performance of the experiment is verified on a fully 13C, 15N-labeled 34-nt hairpin RNA comprising typical structure elements.

Keywords RNA resonance assignment · HCP · Selective pulses · Four-dimensional NMR · Non-uniform sampling

With the advent of several new classes of non-coding RNAs (e.g. siRNA, miRNAs) research has been heavily focused on understanding the role of RNA in cellular processes during normal and diseased states (Esteller 2011), through exploring its structure–function relationship (Briones et al. 2009; Mercer et al. 2009). Over the years, in conjunction with isotope labeling techniques, several NMR approaches (Varani et al. 1996; Wijmenga and van Buuren 1998; Furtig et al. 2003; Flinders and Dieckmann 2006) proved to be highly useful in expanding our knowledge about RNA structure, its basic structural motifs, catalysis and interactions with small molecules or proteins. However, precise structural determination of even moderately sized RNAs can still be problematic. In addition to low proton density in RNAs, these biopolymers comprise only four different nucleotides. Effectively, chemical shift dispersion is inherently low, which entails severe spectral overlaps. For non-coding RNAs a frequent lack of base stacking results in even increased crowding in the NMR spectra. Moreover, similar chemical shifts are observed for many nucleotides having similar chemical environment in helical secondary structures. Recently, automated assignment approach involving no isotope labeling (Aeschbacher et al. 2013) was proposed that requires peak lists from 2D TOCSY, 2D NOESY and natural abundance 1H–13C...
HSQC spectra. However, difficulty may arise while assigning regions/nuclei with irregular or limited statistics. In addition, chemical shift degeneracy or low dispersion still remain a bottleneck for such approaches. This suggests that new high dimensional techniques, resembling 4D/5D methods employed for intrinsically disordered proteins (Zawadzka-Kazimierczuk et al. 2012; Stanek et al. 2013b; Bermel et al. 2013) can be explored for the assignment of poorly resolved resonances in RNAs.

The sequential resonance assignment in RNA is usually achieved using through-space NOE-type (Nikonowicz and Pardi 1993) and/or through-bond HCP (Marino et al. 1994) experiments. The efficacy of both types of experiments is severely affected due to spectral crowding and overlaps, which increases dramatically with the size of RNA. To increase the peak resolution, experiments having HCP concatenated with HCCH-TOCSY were also proposed (Marino et al. 1995; Ramachandran et al. 1996), however, their application remained limited due to significant relaxation losses during TOCSY mixing time and limited resolution achieved using through-space NOE-type (Nikonowicz and Pardi 1993) and/or through-bond HCP (Marino et al. 1994) experiments. For example, the 3D HCP experiment, which provides sequential connectivity via intervening 31P nuclei, i.e. correlating H4′(i)–C4′(i)–P(i) with P(i)–C4′(i–1)–H4′(i–1), has been successful in many applications. However, it suffers from severe spectral overlaps (see Fig. S1a–d in Supplementary Materials) and relies on quite poor resolution of 31P dimension. In principle the peaks can be resolved in 31P dimension but in practice it is limited by its low chemical shift dispersion (≈1.8 ppm) (see Fig. S1e), which makes unambiguous assignment of peaks a challenging task even for moderately sized RNAs. Additionally, in this experiment it is very difficult to unambiguously assign intra- and inter-nucleotide peaks (see Fig. S1b, c), especially when it relates to the most crowded, H4′-C4′, region among sugar carbons. The possibility of sequential correlation through other sugar carbons, i.e. C3′(i–1) and C5′(i) is also hampered due to weak 31P–C3′/5′ couplings making such peaks either absent or too weak; the problem is further augmented by peak overlaps and presence of H5'/H5'' doublets. Clearly, more advanced approaches are needed to achieve an unambiguous sequential assignment in RNAs.

To address these issues we have developed a C4′/H4′ selective, four-dimensional HC(P)CH experiment with “out and stay” type transfer. The experiment includes chemical shifts evolution of 1H4′s and 13C4′s of the adjoining nucleotides thereby linking them in a single experiment with higher peak resolution. The experiment provides sequential connectivities via H4′(i)–C4′(i)–C4′(i–1)–H4′(i–1) correlation. The 31P dimension (e.g. used in 3D HCP) is replaced with better dispersed nuclei, C4′s (≈5 ppm) to improve resolution and alleviate ambiguities during assignments. Multiple quantum (MQ) line narrowing effect (Grzesiek et al. 1995) is implemented to improve the sensitivity of the experiment. In the proposed experiment the intra-nucleotide peaks are efficiently suppressed whereas the inter-nucleotide peaks are enhanced. Different settings of the coherence transfer delay allow for suppression of intra-nucleotide peaks. In the cases where intra-nucleotide peaks are partially suppressed, the opposite signs of two types of peaks still make it convenient to assign them separately without any ambiguities. The experiment employs C3′/C5′ selective inversion pulse to prevent the signal modulation due to 13C–13C homonuclear couplings, these pulses also indirectly enforce the C4′/H4′ selectivity. The schematic design of the experiment is illustrated in Fig. 1 where it also compares the differences from 3D HCP experiment. Figure 1a describes the pathways for generation of both intra and inter-nucleotide peaks in 3D HCP experiment and almost unidirectional flow of magnetization due to suppression of intra-nucleotide peaks in C4′/H4′ selective 4D HC(P)CH experiment. Between the adjoining nucleotides the magnetization on 31P is forward transferred not only to desired C4′s, but also to other weakly coupled carbon spins, i.e. C3′ and C5′. This, collectively, causes a significant loss in sensitivity resulting in weak or undetectable resonances; such feature is not affordable in a 4D experiment. To eliminate these deleterious effects we utilized the C4′ selective inversion pulse during the coherence transfer in the experiment. Figure 1b shows a comparative illustration of the non-selective transfer in 3D HCP with the selective transfer in 4D HC(P)CH.

The pulse scheme for C4′/H4′ selective 4D HC(P)CH experiment is shown in Fig. 2. The experiment is designed with an emphasis on achieving higher resolution with minimum sensitivity losses. High dimensionality is achieved by incorporating three indirect chemical shift evolution periods into the sequence. The pulse sequence (see Fig. 2) comprises two 1H–13C MQ periods (MQ1 and MQ2; storing MQ coherences for most of the period) and a middle 31P–13C single quantum transfer period (SQ). The magnetization flow scheme is as follows:

\[
1H4′(t_1) \xrightarrow{1J_C'N} 13C4′(t_2) \xrightarrow{3J_{CP}} 31P \xrightarrow{3J_{CP}} 13C4′(t_3) \xrightarrow{1J_{CN}} 1H4′(t_4)
\]

Since, as it was shown earlier for nucleic acids (Fiala et al. 1998, 2000), the dominant 1H–13C dipolar relaxation mechanism is significantly attenuated for zero- and double-
quantum coherences, MQ coherences are preserved during the frequency labeling of both C4' evolution periods. In the first MQ1 period (see Fig. 2), the coherence starts from H4' in sugars and is transferred to C4' via non-refocused INEPT. H4' and C4' are then brought into a MQ state and the shared-evolution of chemical shifts of H4' (t1) and C4' (t2) is performed in a constant-time manner by shifting the corresponding hard 180° pulses within the MQ1 period. In order to evolve C4'–P couplings and achieve a coherence transfer, a 180° pulse on 31P is applied simultaneously with the C4' inversion pulse on 13C channel. During this period (τb), the evolution due to homonuclear carbon coupling (C4'–C3' and C4'–C5') is refocused by two cosine modulated IBURP-2 (Geen and Freeman 1991) pulses (P in Fig. 2) which selectively and simultaneously invert the frequency bands of C3' and C5' ribose sugar carbons. Since C2' carbons share the same spectral region as of C3', inversion of later also inverts the C2' carbons. Effectively, the use of inversion pulses lead to an indirect selection of C4' and hence H4' during the MQ1 period. The next 90° pulse on H4' and Δ delay refocus the C4' anti-phase to H4' whereas the subsequent 90° pulses on C4' and 31P transfer the coherence onto 31P.

In the next SQ period magnetization on 31P is brought into transverse plane and 31P–13C couplings are evolved. In the middle of this period a C4' selective cosine modulated IBURP-2 pulse (Q in Fig. 2) is employed to prevent dephasing due to P1–C3' and P1–C5' couplings and achieve selectivity for P → C4' transfer.

The evolution during the delay τb refocuses the P1–C4i anti-phase and creates the P1–C4i–1 anti-phase operators, and determines the suppression of intra-nucleotide peaks or enhancement of inter-nucleotide peaks. The suppression level of intra-nucleotide peaks is a trade-off between the J-coupling optimum delay τb and relaxation. The intensity of an intra-nucleotide peak is proportional as:

$$I_{\text{intra}}(C4'_{i-1}P_iC0') \propto r \cos\left(\frac{3\pi}{2} J_{C4'_{i-1}P_iP_i} \tau_b\right) \cos\left(\frac{3\pi}{2} J_{P_iC4'_{i-1}P_i} \tau_b\right) e^{-Rb}$$

whereas that of inter-nucleotide peak is related as:

$$I_{\text{inter}}(C4'_{i-1}P_iC0') \propto r \sin\left(\frac{3\pi}{2} J_{C4'_{i-1}P_iP_i} \tau_b\right) \sin\left(\frac{3\pi}{2} J_{P_iC4'_{i-1}P_i} \tau_b\right) e^{-Rb}$$

where $R$ is 31P SQ transverse relaxation rate and $r$ incorporates the contributions from all other passive couplings.

The coherence transfer efficiency and hence the intensities are dependent on $J_{C4', P_i}$ values (discussed later in the text). The experiments are performed at various $J$ values, however at ~10 Hz we found the least loss of number of resonances in the spectrum. The transverse relaxation rate ($R$) is estimated experimentally for 31P and intensities for both types of peaks are plotted (see Fig. S2 in Supplementary Materials), with increasing transfer delay (τb).

A suitable delay (~38 ms for this study) is chosen to maximize the inter-nucleotide peak intensities, which in turn also minimizes the intra-nucleotide terms, especially in the cases where $J_{C4', P_i} \approx J_{P_iC4'_{i-1}}$.  

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**Fig. 1** A schematic and comparative illustration of magnetization transfer in C4′/H4′ selective 4D HC(P)CH experiment. Red and blue paths represent the magnetization flow in 3′→ 5′ and 5′→ 3′ directions, respectively. The numbers in circles represent the coherence transfer steps leading to cross-peaks whereas the suffix “a” inside circles represents a path which generates intra-nucleotide peaks. In a 3D HCP experiment magnetization flow splits from 31P, generating both intra- and inter-nucleotide peaks whereas in 4D HC(P)CH experiment (a) intra-nucleotide peaks are suppressed (denoted by dotted red/blue arrows) and involving mostly unidirectional flow of magnetization. (b) Illustrates other key differences in coherence transfers between 3D HCP and 4D HCPCH experiments. In 3D HCP the magnetization on 31P gets forward transferred (P → C3′/ C5′, orange/green solid arrows) to other sugar carbons (C3′ and C5′) whereas in 4D HCP(P)CH experiment such pathways are blocked (denoted with cross on orange/green arrows); again the suppressed intra-nucleotide peak is shown by dashed red arrow. For the interpretation of colors in this figure the reader is referred to the online version of the Journal.
In the consecutive block MQ2, the coherence is forward transferred to C4′(i) where its chemical shifts are indirectly recorded (t3) preserving the MQ coherences similarly to the MQ1 block in the sequence. During the same period (t3) refocusing of P–C4′ couplings is also achieved by application of a 180° pulse on 31P in synchrony with the moving 180° pulse on 13C channel. Another 180° pulse is centrally placed on 1H channel to refocus its chemical shift evolution. 13C adiabatic composite pulse on 13C channel. Another 180°/2 pulses were 7.8, 18.1 and 26.5 μs for 1H, 13C and 31P, respectively. Proton carrier frequency was set on resonance with water (4.68 ppm), carbon carrier was set to the centre of 13C′s (83.00 ppm) and 31P carrier was set to −4.10 ppm. Quadrature detection in t1, t2 and t3 is accomplished by altering φ1, φ2 and φ5, respectively, according to the States-TPPI procedure. 16-step phase cycle is as follows: φ1 = x; φ2 = z, −z, φ1 = z; φ2 = z, −z, φ1 = 2(y), 2(−y); φ3 = 4(x), 4(−x); φ5 = 8(y), 8(−y) and φ5 = y, 2(−y), y, 2(y), −y. Delays are set as follows: Δ = 3.5 ms ≈ (2 JCH)−1, t5 = t5 = 20.9 ms and t5 = 38 ms. Gradient levels and durations are: G1 (0.2 ms, 12.7 G/cm), G2 (0.8 ms, 33.7 G/cm), G3 (1.0 ms, 42.5 G/cm), G4 (2.0 ms, 15.61 G/cm) and G5 (0.5 ms, 4.6 G/cm). A total of 1,300 (~9%) sampling points (t1, t2, t3) were randomly chosen from a 31 × 22 × 22 Cartesian grid according to Gaussian probability distribution, p(t) = exp(−(t/tmax)2/2σ2), σ = 0.5, with Poisson disk restrictions (Kazimierczuk et al. 2008). Maximum evolution times of 20 (t1max), 14 (t2max) and 14 ms (t3max) were achieved in the indirectly detected dimensions. Acquisition time was set to 85 ms (t4max). Spectral widths of 15 (ω1), 15 (ω2), 15 (ω3) and 12 kHz (ω4) were assumed. The total experiment duration was 75 h. The interscan delay of 1.8 s for optimal recovery of 1H magnetization (sensitivity per unit time) was used. The experiment was performed at 298 K on the Agilent DDR2 600 MHz spectrometer equipped with a room-temperature penta (1H/13C/15N/2H/31P) probe.

High dimensional experiments, 5D HCP-CCH COSY (Krahnenhul et al. 2014) and 4D-NUS C(αr,o) C(rib) NOESY (Stanek et al. 2013a), dedicated for sequential resonance assignment in RNAs.

To achieve higher dimensionality with reasonable resolution in the indirectly detected dimensions, non-uniform sampling (NUS) was employed. Using NUS we are able to acquire 4D HC(P)CH experiment with high evolution times: 20 ms (τ1), 14 ms (τ2) and 14 ms (τ3). The processing of 4D NUS data was accomplished by the home-written software package Signal Separation Algorithm (SSA) (Stanek et al. 2012), which can be downloaded free of charge for non-commercial purposes from the website http://nmr.cent3.uw.edu.pl.

We have tested the performance of C4′/H4′ selective 4D HC(P)CH experiment on a fairly demanding RNA sample which encompasses typical structural elements. The experiments were run on a 13C,15N-labeled 34-nt hairpin RNA (1.5 mM in D2O solution) consisting of two A-RNA form stems, one adenine bulge, an asymmetric internal loop and a GAAA terminal loop (Cevec et al. 2010). The
4D HC(P)CH spectrum was easily analyzed with SPARKY (Goddard and Kneller 2004) program by synchronizing two dimensions (H4 and C4) of ith nucleotide (see Figs. 3, S3 in Supplementary Materials); the resulting 2D plane consists of inter-nucleotide peaks, i.e. to the (i−1)th and (i+1)th nucleotides. In other words, to achieve the sequential assignment, H4/C4 plane of one nucleotide is correlated with the H4/C4 planes of two neighboring nucleotides. Figures 3, S3 show the representative 2D planes of 4D HC(P)CH spectrum illustrating the resolution advantage in the experiment. It can clearly be seen that heavily overlapped peaks (e.g. C33, U32, C31, A28, C29, A20, G19) in 2D 13C-HSQC (Fig. 3a) are clearly resolved in 4D experiment along the H4/C4 planes of adjoining nucleotides (Figs. 3b–d, S3a–d). To compare the suppression levels of intra-nucleotide peaks, another 4D experiment was acquired without emphasis on suppression, i.e. using $\tau_s \sim 22$ ms. The 2D planes from this version of
experiment consist of one intra- and two inter-nucleotide peaks (of course, with an exception for terminal nucleotide). As can be expected, in this version of experiment intra-nucleotide peaks are more intense than inter-nucleotide peaks (see Fig. 3e–g). A significant suppression of intra- and enhancement of inter-nucleotide peaks can be compared between the two versions of experiment in Fig. 3 (b, e), (c, f) and (d, g) pairs. In the cases of incomplete suppression of intra-nucleotide peaks, their opposite sign still reduces ambiguities during assignments.

Overall, 19 sequential connectivities were successfully established (see Fig. 4) using C4’/H4’ selective 4D HC(P)CH whereas 3D HCP experiment could provide only 4 sequential links in 34-nt RNA. Comparatively, the previously reported 4D C_(aro)-C_(ribo)-NOESY experiment provided 17 sequential links, which reflects the difficulty of the investigated RNA sample. Interestingly, 4D HC(P)CH and 4D NOESY experiments provided complementary data for sequential assignment. In combination, 26 (out of 33) sequential links were successfully assigned. The missing assignments are either due to structural mobility, manifesting in enhanced relaxation during coherence transfer periods or due to small C4’–P couplings. In our previous study we have shown that, in this RNA, the asymmetric internal loop adopts two energetically comparable families of structures, which both satisfy NMR data (Cevec et al. 2010). In addition, the C4’ → P/P → C4’ transfers in 4D HC(P)CH experiment rely on the C4’–P couplings (\( J_{C4',P} \)), which in turn depend on the \( \beta/\epsilon \) torsional angles in RNA (Schwalbe et al. 1994; Legault et al. 1995; Hu et al. 1999).

For RNA used in this study (PDB ID: 2KPV) the coupling constants (\( J_{C4',P} \)) were calculated based on the parameterized Karplus equation (Mooren et al. 1994). It can be observed that for some of the cases the C4’–P couplings are very small (see Fig. 5) and an efficient coherence transfer is difficult to achieve. It is noteworthy that, in this study, most of the missing resonances relate to the internal loop or to the proximate residues where the \( \beta/\epsilon \) angles are large and therefore C4’–P couplings are very small.

To conclude, we have introduced a through-bond, C4’/H4’ selective, non-uniformly sampled 4D HC(P)CH experiment for sequential assignments in RNAs. The incorporated indirect dimensions, along with the replacement of evolution of \( ^{31}\text{P} \) by \(^{13}\text{C}4'\), significantly enhanced the spectral dispersion. NUS was employed to achieve high

![Fig. 5](image-url)
resolution in all the indirectly detected dimensions. Band selective inversion pulses were used to prevent signal modulation due to C4'-C3' and C4'-C5' couplings and to indirectly select the C4'/H4' region. Experiment involved the suppression of intra-nucleotide peaks, as a result, the number of ambiguities were further reduced. We have demonstrated that the C4'/H4' selectivity and attenuated relaxation of MQ coherences partially compensated the sensitivity losses entailing the increased dimensionality. Despite lower sensitivity, the proposed experiment clearly outperforms the conventional HCP experiment, which suffers from critical overlap in the “linking” 31P dimension. The experiment is proposed as a complementary tool to 3D/4D NOE experiments and augments the set of high dimensional experiments aimed at improving resolution and reducing ambiguities during resonance assignments in RNAs with poor chemical shift dispersion.

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